LIGHT AND ULTRAMICROSCOPICAL ASPECTS OF THE *IN VITRO* DEVELOPMENT OF *HEMATODINIUM* SP. (DINOFLAGELLATA) FROM ATLANTIC SNOW CRABS (*CHIONOECETES OPILIO*)

BY

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Abstract

Hematodinium is a parasitic dinoflagellate of numerous crustacean species, including the economically important Atlantic snow crab, *Chionoecetes opilio*. The parasite was isolated from *C. opilio* in the fall of 2010 and 2011 and cultured *in vitro*. Several media were tested, with *Nephrops* medium yielding superior results. Life stages were characterized using light, scanning and transmission electron microscopy. This is the first time *Hematodinium* has been isolated from *C. opilio* and cultured *in vitro,* and is only the second *in vitro* study on *Hematodinium* which used electron microscopy.

Numerous life stages were observed: amoeboid trophonts, sporonts, sheet-like and arachnoid coenocytes, dinospores, post-dinospores, and schizonts. In crab hemolymph, amoeboid trophonts and sporonts were observed. In cultures that were seeded with sporonts, further progression to motile dinospore stages occurred; however, those seeded with amoeboid trophonts did not progress to dinospores, often remaining as amoeboid trophonts or occasionally forming schizonts. Additionally, two types of coenocytes were associated with sporonts: sheet-like and arachnoid coenocytes. Only sheet-like coenocytes were associated with trophonts.

Trophonts that were morphologically different were ultrastructurally similar, thus no distinction between filamentous and amoeboid trophonts was made. Ultrastructurally, trophonts possessed amphiesmal vesicle membranes, nuclei with permanently condensed chromosomes, mitochondria with tubular cristae, lipid droplets, and lipofuscin granules. Lipofuscin granules were characterized by their autofluorescence and ultrastructural similarity to lipofuscin granules found in other invertebrates and vertebrates. Additionally, lipofuscin granules appeared to degranulate or to be expelled from the cell

during *in vitro* cultivation. The exact mechanism of this is unknown. Sporonts possessed all the features noted for trophonts and also had trichocysts. Mature trichocysts were long, rectangular shaped organelles with a width of 223 ± 2.11 nm (n=100) and of unknown length. Trichocyst development was examined ultrastructurally. Developing trichocysts were associated with the Golgi apparatus and first appeared as large vesicles containing a homogeneous, granular material. During development, the granular material condensed to form a crystalline core. Mature trichocysts were characterized by an electron dense crystalline core surrounded with a tightly juxtaposed single membrane.

Early sporonts appeared similar to amoeboid trophonts in morphology; but became more spherical prior to dinospore formation. Late sporonts divided into four daughter cells, indicating a significant replicative step. Dinospores shared all ultrastructural features of sporonts, but possessed two flagella: one transverse and one longitudinal for motility. All dinospores were morphologically identical and resembled the macrodinospores observed in other *Hematodinium* spp. No microdinospores were observed; this lack of detection is likely due to the low number of observed cultures that progressed to the dinospore stage. After ~ 2 weeks *in vitro*, dinospores lost their flagella, and became more spherical. Furthermore, the nuclear structure was dissimilar to other stages, with beaded chromatin only evident along the periphery of the nucleus in addition to a single bead in the centre, rather than ubiquitous throughout the nucleus. Thus these were termed ‘post-dinospores’. Additionally, schizonts were noted as very large cells with large, relatively empty lipofuscin granules. Schizonts were only seen in contaminated or old cultures, and were thus considered as an abnormal form. Notably, no viable gorgonlocks or clump colonies were observed as noted in *Hematodinium* sp.

from other crustacean hosts. In the present study, unique filopodia-like structures were observed protruding from early sporonts and trophonts and attached to neighbouring cells.

Cryopreservation of *Hematodinium* was also attempted on sporonts and trophonts. Although recovery rates were very low, viable *Hematodinium* survived ~ 3 months in cryostorage. Two cryoprotectants, 3% glycerol and 10% DMSO, yielded positive results. This is the first successful cryopreservation of *Hematodinium*.

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Dedication

I dedicate this thesis to my beautiful wife, Alison, who was always there to support and encourage me. She was there when times were tough and she helped me to push through. I do not think I would have completed this work if not for her guiding hand and supportive words.

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List of Abbreviations

ASW- Artificial sea water
AVC- Atlantic Veterinary College
BCD- Bitter crab disease
dH_2O- Distilled water
DMEM- Dulbecco's modified Eagles medium
DMSO- Dimethyl sulfoxide
EtOH- Ethanol
FITC- Fluorescein isothiocyanate
PO- Propylene oxide
PPT- Parts per thousand
RT- Room temperature
SEM- Scanning electron microscopy
TEM- Transmission electron microscopy
WHO- World Health Organization

Chapter 1: GENERAL INTRODUCTION

1.1 Overview

Hematodinium is a parasitic dinoflagellate of crustaceans. It was first discovered off France in 1931 in the European shore crab, *Carcinus maenas*, and the harbour crab, *Liocarcinus depurator*. Since its discovery, it has spread worldwide infecting a wide range of crustaceans, many of which are economically important (Chatton and Poisson, 1931; Newman and Johnson, 1975; Maclean and Ruddell, 1978; Meyers *et al*., 1987; Latrouite *et al*., 1988; Wilhelm and Boulo, 1988; Hudson and Lester, 1994; Hudson and Shields, 1994; Messick, 1994; Taylor and Khan, 1995; Wilhelm and Mialhe, 1996, Ryazanova *et al*., 2010). There have been several outbreaks of *Hematodinium* induced disease in snow crab, *Chionoecetes opilio*, Tanner crab, *C. bairdi*, Norway lobster, *Nephrops norvegicus*, and blue crab, *Callinectes sapidus* with prevalences reaching 20-100% in some locations (Meyers *et al*., 1987; Field *et al*., 1992; Messick, 1994; Mullowney *et al*., 2011). Furthermore, *Hematodinium* infections in crustaceans are often lethal (Meyers *et al*., 1990; Messick and Shields, 2000; Stentiford and Shields, 2005). Within the Atlantic Canadian region, the parasite is considered a significant threat to snow crab populations (Bower, 2013). Prevalence varies, but has reached up to 30% off Newfoundland and Labrador at inshore regions (Mullowney *et al*., 2011). With Atlantic snow crab landing values of over $280 million annually, *Hematodinium* poses a threat to this lucrative fishery and the regional economy.

1.2 Taxonomy and general dinoflagellate characteristics

Hematodinium is a member of the Syndiniales taxon (fourth rank) within the Dinoflagellata taxon (third rank) within the Dinozoa taxon (second rank) within the

Alveolata taxon (first rank) within the eukaryotic super-group Chromalveolota (Adl *et al*., 2005).

The Syndiniales, including *Hematodinium,* apparently diverged earlier in evolutionary history from other dinoflagellates, due to their resemblance to more typical eukaryotic cells (Jackson *et al*., 2012). Syndiniales are unique dinoflagellates in that they lack both thecae and plastids, while they have significantly less genetic material, possess histones, and are obligate marine parasites (Taylor, 1987; Guillou *et al*., 2008). The genus *Hematodinium* currently has two species, *H. perezi* (Chatton and Poisson, 1931), and *H. australis* (Hudson and Shields, 1994). There have also been many reports of *Hematodinium* sp. and *Hematodinium*-like organisms that have not been characterized to the species level. There is speculation regarding the distinctiveness of the two species of *Hematodinium* since no molecular analysis was used to differentiate the species. Morphologically, *H. australis* differs from *H. perezi* based on the size of the vegetative stage, the nuclear structure, and plasmodium life stages and its austral location (Hudson and Shields, 1994), but there has yet to be any molecular sequence data collected for *H. australis*. The original descriptions of *H. perezi* were hand drawn diagrams, and only recently has molecular and morphological characterization of *H. perezi* occurred (Small *et al*., 2012).

Hudson and Adlard (1996) proposed the classification of two new species for *Hematodinium* derived from the snow crab, *Chionoecetes opilio* and from the Norway lobster, *Nephrops norvegicus*, whereas Hamilton *et al*. (2010a) concluded that various 'clades' of *Hematodinium* sp. exist in the North Atlantic, with some clades being host-specific while others are generalists. Since current molecular assays can detect

Hematodinium at the genus level (Gruebl *et al*., 2002; Small *et al*., 2006, 2007; Jensen *et al*., 2011), future research should focus on classifying *Hematodinium* sp. and *Hematodinium*-like organisms to the species level by using a combination of morphological and molecular methods.

Dinoflagellates, including *Hematodinium* sp., have at least one life stage where it has two flagella, one transverse and one longitudinal, typically in a dinokont arrangement. The transverse flagellum provides propulsive force whereas the longitudinal flagellum directs the organism. Furthermore, dinoflagellates have permanently condensed chromosomes (dinokaryon), and extra-nuclear spindles; their nuclear membrane remains intact during mitosis, and many posess thecae (cellulose plates) (Taylor, 1987). Other features used to morphologically characterize dinoflagellates are their cortical alveoli and mitochondria containing tubular cristae. For thecated dinoflagellates, the tabulation pattern of theca is the primary means of classification.

Hematodinium sp., like most dinoflagellates, possesses extrusomes. Extrusomes are organelles (trichocysts, mucocysts and nematocysts) that are ejected from the organism and may serve predatory or defensive roles (Taylor, 1987). Mucocysts are flask-shaped, and are associated with the theca. Although their function is largely unknown, they release a granular substance which is considered to be the source of mucoid cysts found in some unthecated dinoflagellates (Taylor, 1987). Nematocysts are complex organelles in some dinoflagellates, and although their role is not completely understood, they are assumed to aid in food capture.

Although, trichocysts are common extrusomes in dinoflagellates, they have been more extensively studied in ciliates, especially *Paramecium* sp. (Bannister, 1972; Hausmann, 1978). Extrusion of trichocysts occurs explosively, however the mechanism of action is unknown. Although the functional role of trichocysts is considered congruent among alveolates, dinoflagellate trichocysts differ morphologically from ciliate trichocysts. The most notable differences are found in the base and shaft of trichocysts; ciliates have a wider, pyriform base, whereas in dinoflagellates the base is uniform in width (Lee and Krugens, 1992). Dinoflagellate trichocysts have two sections: an apical end, which has a series of flexible fibres, and a body, which consists mainly of a crystalline core (Bouck and Sweeney, 1966).

Trichocysts have been found in *Hematodinium* and are used as one of the main criteria for distinguishing between sporonts and trophonts (Meyers *et al*., 1987; Appleton and Vickerman, 1998). To date however, there have been no studies investigating the ultrastructure or development of trichocysts in *Hematodinium*. Therefore, characterizing both the development and morphology of trichocysts may be important for defining life stages and for understanding their function in this parasitic dinoflagellate.

1.3 Life cycle of Hematodinium

An understanding of the complete life cycle and behaviour of *Hematodinium* remains elusive and more research is needed in this area. Three life stages of *Hematodinium* are widely accepted: the trophont (vegetative stage), the sporont (non-vegetative stage), and the dinospore (mastigote/motile stage) (Figure 1.1). More life stages have been characterized *in vitro* through extensive life cycle studies of *Hematodinium* isolated from the Norway lobster, *N. norvegicus* and from the blue crab, *C. sapidus* (Appleton and

Vickerman, 1998, Li *et al*., 2011; Figure 1.2; 1.3). Trophonts are characterized by rapid development, may be uni- or multi-nucleated, often contains vacuoles displaying a foamy cytoplasm and lack flagella and trichocysts. The sporont is uninucleate, possesses trichocysts and lacks flagella. The appearance of trichocysts marks the transition from trophont to sporont; however, no information on the metabolic and replicative rates of cells is available to distinguish between stages (Meyers *et al*., 1987; Appleton and Vickerman, 1998; Stentiford and Shields, 2005: Li *et al*., 2011). Also, in the description of *H. australis*, trichocysts are recognized in the trophont stages (Hudson and Shields, 1994). This brings into question 1) the legitimacy of using trichocysts as life stage defining features or the possibility that 2) the descriptions of *H. australis* trophonts were transitionary stages. Others distinguish life stages based on nuclear to cytoplasmic ratios; differentiation based on this criterion, however, lacks precision and is subjective (Meyers *et al*., 1987). Clearer definitions of *Hematodinium* life stages are needed to properly define *Hematodinium* life stage transitions.

Extensive *in vitro* studies have revealed additional life stages of *Hematodinium* isolated from *N. norvegicus* and *C. sapidus* (Appleton and Vickerman, 1998; Li *et al*., 2011). Appleton and Vickerman (1998) described morphological characters using both light and electron microscopy from *Hematodinium* isolated from *N. norvegicus*. Four trophont forms were found within *in vitro* cultures derived from *N. norvegicus*: filamentous trophonts, clump colonies, gorgonlocks colonies, and arachnoid trophonts. Filamentous trophonts were vermiform in shape and occasionally had flexing motion. When maintained at 8^{o}C, filamentous trophonts grew rapidly and could be maintained indefinitely when sub-cultured at biweekly intervals. Gorgonlocks colonies were a

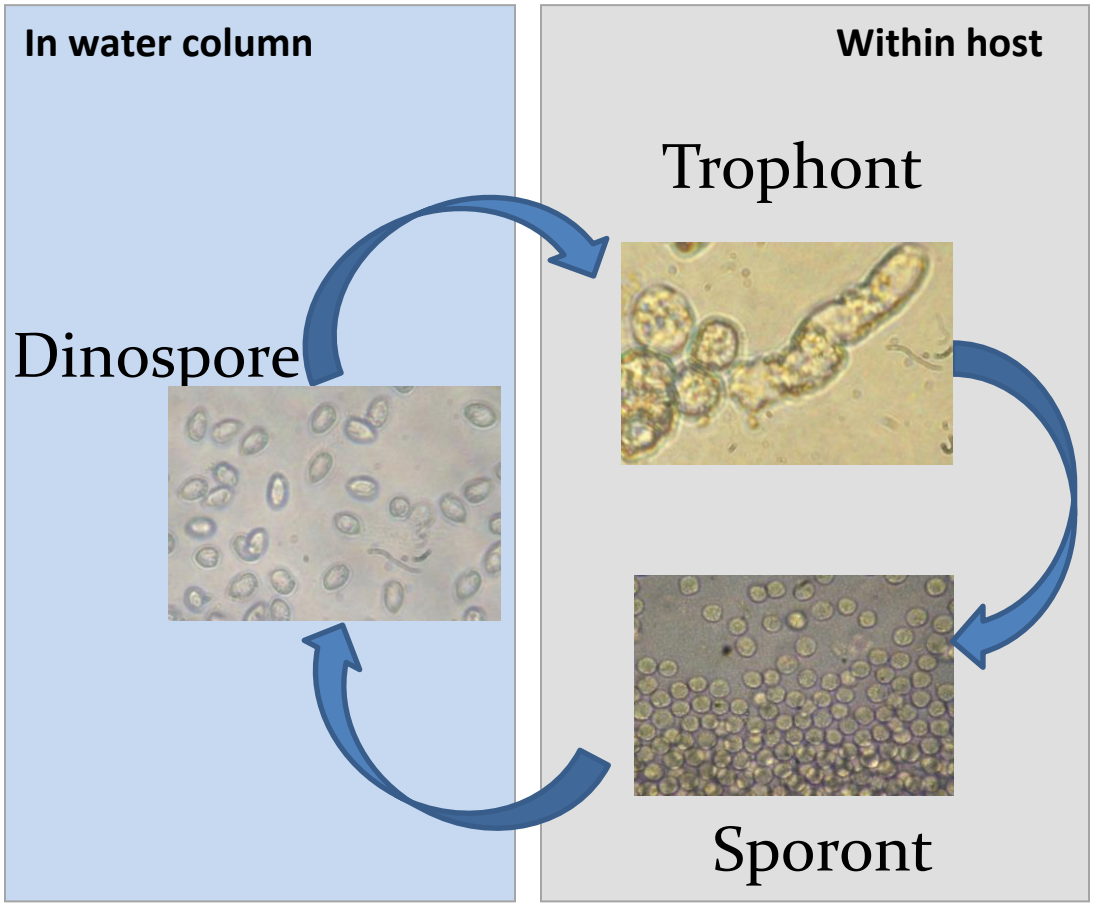

Figure 1.1: Simplified life cycle diagram highlighting the three main life stages of *Hematodinium* sp.

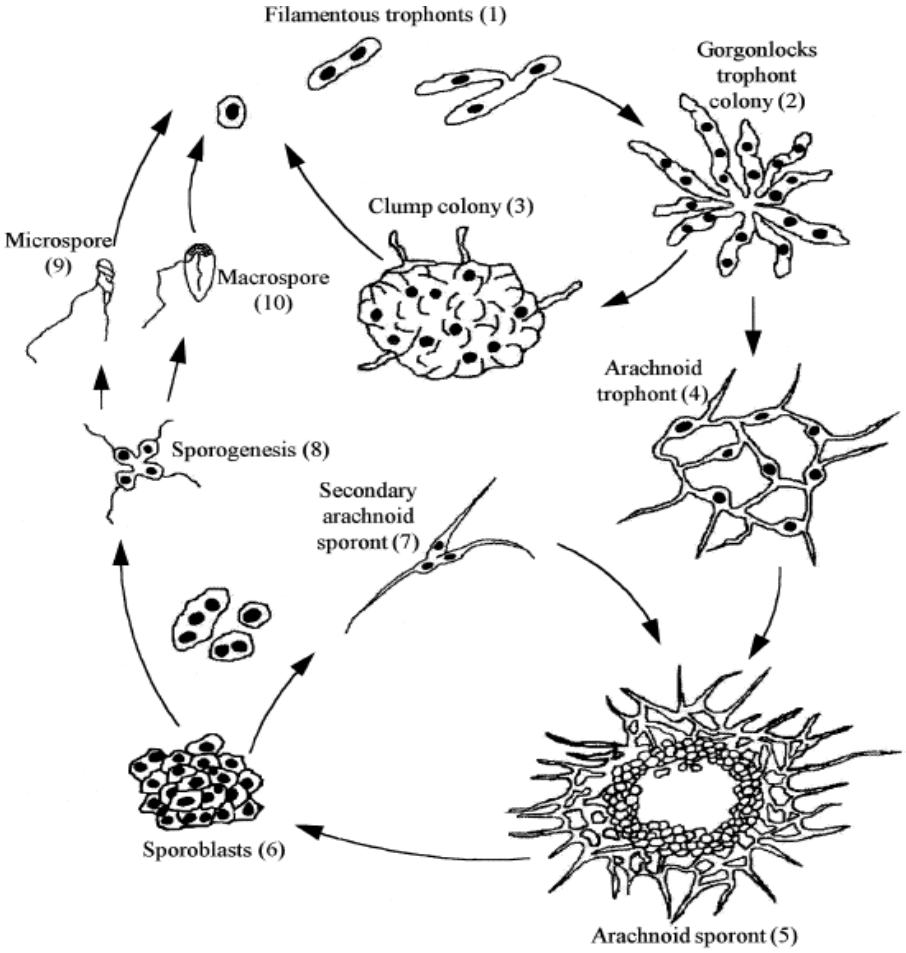

Figure 1.2*: In vitro* life cycle of *Hematodinium* sp. isolated from *Nephrops norvegicus* proposed by Appleton and Vickerman (1998)

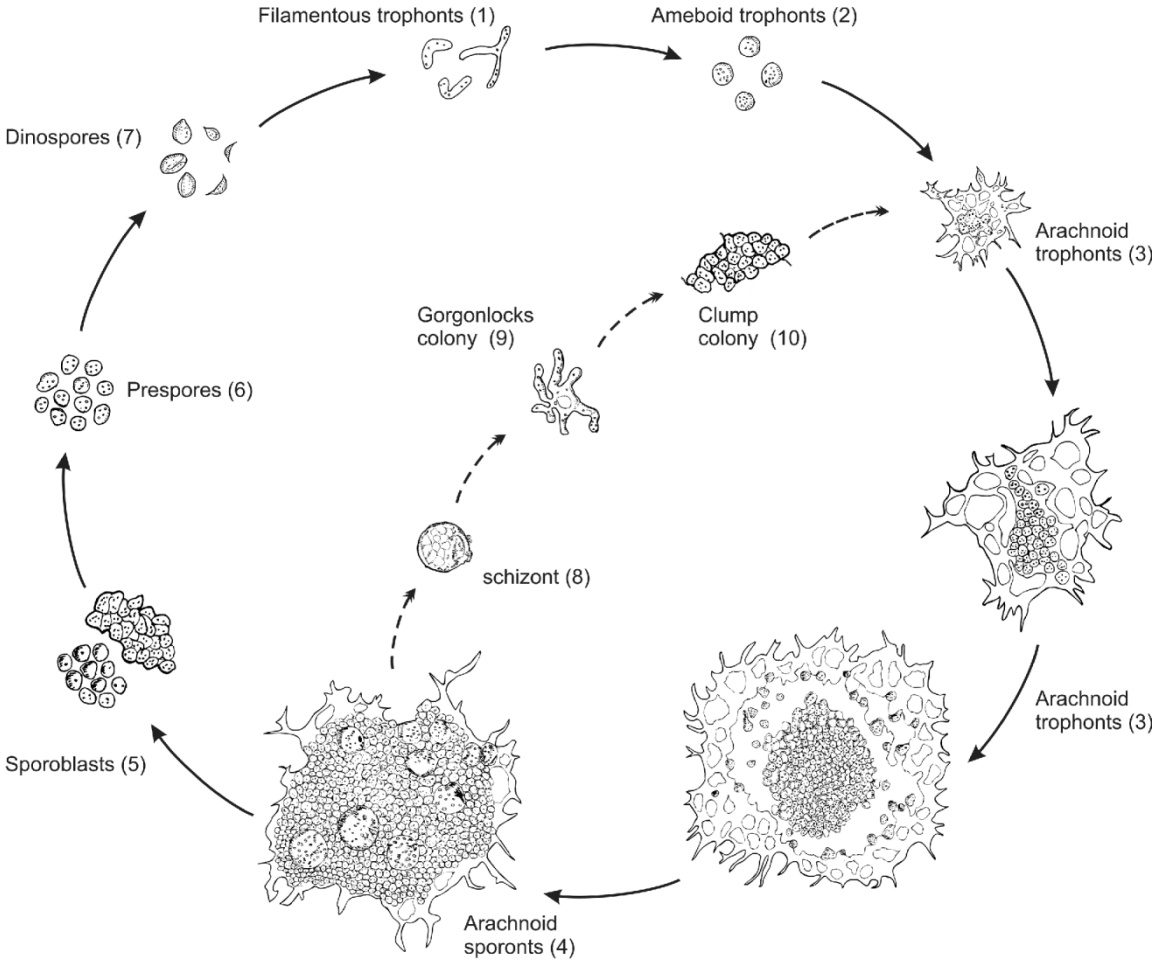

Figure 1.3*: In vitro* life cycle of *Hematodinium* sp. isolated from *Callinectes sapidus* proposed by Li *et al*. (2011)

variation of multinucleate filamentous trophonts; progression to clump colonies occurred when gorgonlocks colonies were not sub-cultured. Clump colonies were large masses of interdigitized syncytia that became senescent unless sub-cultured to fresh medium at lower densities, where they would revert to filamentous trophonts. Gorgonlocks, when kept in fresh medium, developed to arachnoid trophonts. Arachnoid trophont and arachnoid sporont stages were complex syncytial networks attached to the substratum. Ultrastructurally, cytoplasmic structures of arachnoid stages closely resembled filamentous trophont stages (vacuolated cytoplasm, peripheral electron-dense inclusions, and many lipid globules). A sporogenic mass developed near the centre of the cell mass in arachnoid sporonts, differing from arachnoid trophonts. Cultures of arachnoid trophonts transformed into arachnoid sporonts and subsequently to sporoblasts. Sporoblasts were produced from the centre of the arachnoid sporont structure and formed a raised sporogenic mass. Each sporoblast gave rise to four dinospores. Sometimes single sporoblasts would be released from the arachnoid structure, and these apparently corresponded to sporoblast stages observed in circulating hemolymph in late stage infections of Norway lobsters (Field *et al*., 1992; Appleton and Vickerman, 1998). Similar stages of *Hematodinium* were identified from *in vitro* cultures derived from *C. sapidus* (Li *et al*., 2011). Notably, Li *et al*. (2011) used light microscopy solely for characterizing life stages. They observed all four trophont stages of Appleton and Vickerman (1998), with one additional form, the amoeboid trophont, which appeared after the filamentous trophont. Arachnoid trophont stages were derived from amoeboid trophont stages and not from gorgonlocks colonies as described by Appleton and Vickerman (1998). Li *et al*. (2011) did observe gorgonlocks colonies and clump colonies

which were transient forms. Gorgonlocks transitioned to clump colonies, similar to results found by Appleton and Vickerman (1998); however, these clump colonies developed into amoeboid trophonts and not into filamentous trophonts.

Arachnoid trophont and sporont stages were similar to those described by Appleton and Vickerman (1998), with the exception that schizont stages occasionally arose from arachnoid sporonts. Schizonts resembled sporoblasts, but were much larger and gave rise to transient gorgonlocks and clump colonies. Arachnoid sporonts gave rise to sporoblasts which ultimately developed to dinospores. Unlike that described by Appleton and Vickerman (1998), there was no replication during sporoblast transition to dinospores in isolates from *C. sapidus* (Li *et al*., 2011).

Dinospores exist as either macro or microdinospores. Macrodinospores are slightly larger than microdinospores (16-20 µm long x 8.5-11.5 µm wide vs. 11-14 µm long x 4.5-6.5 µm wide) and can be difficult to distinguish. Mature macrodinospores have a prominent keel and are bullet shaped, whereas mature microdinospores are corkscrew shaped and have a notable refractile body (Appleton and Vickerman, 1998). Apparently only one type of dinospore is produced from a single isolate or crab (Meyers *et al*., 1987; Appleton and Vickerman, 1998; Stentiford and Shields 2005), although there appears to be an equal ratio of micro and macrodinospores within a population. What induces dinospore dimorphism is unknown (Appleton and Vickerman, 1998; Li *et al*., 2011). Sporonts and dinospores are only observed in late stages of infection (*in vivo*), thus depletion of metabolic reserves in combination with changes in host biochemistry may influence transition (Schmidt and Platzer, 1980; Stentiford *et al*., 2000). Additionally, as *Hematodinium* attains higher densities in culture, transition to dinospores

occurs. This may reflect the depletion of nutrients within the medium (Appleton and Vickerman, 1998; Stentiford and Shields, 2005; Li *et al*., 2011).

Life cycle stages observed within *in vitro* cultures may not represent natural *Hematodinium* life stages. Since cell culture lacks natural tissue architecture, some of the life stages observed *in vitro* may be artefacts. Furthermore, in *H. perezi* infections of *C. sapidus*, *Hematodinium* occasionally attached to the wall of hemolymph vessels as multinucleated forms (Small *et al*., 2012). Thus, many of the more complex stages observed *in vitro* may be attached to tissues *in vivo*, possibly in subclinical infections. Life stages seen in *Hematodinium* isolates may also vary between different host species, as the parasite may be host adapted or vary based on environmental conditions. *In vitro* life stages of *Hematodinium* isolated from *N. norvegicus* appear to resemble those from *C. sapidus*, although there are some significant differences. Notably that the gorgonlocks and clump colonies appear to be part of an alternative replicative pathway; additionally individual sporoblasts did not give rise to four dinospores in isolates from *C. sapidus* (Li *et al*., 2011). Furthermore, distinguishing between some stages (i.e. clump colonies vs. sporoblasts) using solely light microscopy is difficult (Li *et al*., 2011).

Further research describing *Hematodinium* life stages would benefit from using both electron and light microscopy. To date, only one study has described the ultrastructure of *Hematodinium* cultured *in vitro* (Appleton and Vickerman, 1998). Further studies confirming and replicating previous findings, using *Hematodinium* isolates from other crustaceans, would provide corroborative evidence for our understanding of *Hematodinium*'s life cycle, ultrastructure, and behaviour.

1.4 Pathophysiology of Hematodinium infections

Studies have investigated disease progression of *Hematodinium* revealing that the parasite is usually first observed in hemal spaces associated with the hepatopancreas (Field and Appleton, 1995; Sheppard *et al*. 2003). Later, *Hematodinium* is associated with numerous organs including gills, heart, and skeletal muscle as it spreads systemically. *Hematodinium* is not found intracellularly and is usually restricted to the hemal spaces; however it may be found within the tissue architecture in late stages of infection (Meyers *et al*., 1987; Field and Appleton, 1995; Stentiford *et al*., 2002; Sheppard *et al.*, 2003; Wheeler *et al*., 2007). The occurrence of *Hematodinium* within tissues, however, is likely due to pressure necrosis caused by high densities of parasites resulting in subsequent tissue degradation rather than active parasite invasion. Apparently, organs are already severely impaired when circulating dinoflagellates are in hemolymph (Field and Appleton, 1995). This result likely indicates that most pathological studies have focused on late stages of infections (Meyers *et al*., 1987; Field and Appleton, 1995; Stentiford *et al*., 2002; Sheppard *et al.*, 2003; Wheeler *et al*., 2007).

Dinospores are presumably an infective stage of the parasite. Inside the host, dinospores likely transition to trophonts and rapidly proliferate; however, conclusive evidence that dinospores are the natural transmissible stage of the parasite has not been established (Shields, 1994; Stentiford and Shields, 2005). Since *Hematodinium* shifts from a trophont to sporont/pre-spore stages late in infection (Schmidt and Platzer, 1980; Meyers *et al*., 1987; Stentiford *et al*., 1999; Stentiford *et al*., 2000; Hamilton *et al*., 2010b) and changes in host biochemistry are reported during *Hematodinium* infection, transition could be due to some environmental cue. There may also be attachment of *Hematodinium* to the outer walls of hemolymph vessels (Field and Appleton, 2005; Small

et al., 2012), and this could represent early stages of infection that correlate with the arachnoid stages observed *in vitro*. Other forms, such as gorgonlocks colonies observed *in vitro*, have not been observed *in vivo*. This could reflect that some of these forms found *in vitro* represent early stages during infection. Furthermore, early infections of *Hematodinium* are difficult to diagnose likely contributing to the apparent discrepancy in life stages observed *in vitro* compared with *in vivo* systems. Research surrounding early detection and life stage characterization of these stages is paramount to our understanding of disease pathogenesis and transmission.

1.5 Potential modes of Hematodinium transmission

There has been moderate success in transmission studies of *Hematodinium, as* researchers have injected trophonts, sporonts, dinospores, or infected hemolymph directly into the hemal sinuses of a naïve crustacean (Meyers *et al*., 1987; Eaton *et al*. 1991; Hudson and Shields, 1994; Shields and Squyars, 2000). Although both amoeboid trophonts and dinospores grow and replicate in naïve hosts, the infective stage of the parasite is unknown as direct injection into hemal spaces does not mimic natural conditions. Nevertheless, dinospores have been observed in *C. sapidus* at very high densities (1.6×10^8 dinospores ml^{-1}), and have been released via the gills in the blue crab, *C. sapidus,* Norway lobster, *N. norvegicus*, Tanner crab, *C. bairdi*, and edible crab, *Cancer pagurus*. Dinospores remain viable in seawater longer than other life stages and may represent the infective/dispersal stage in the *Hematodinium* life cycle (Love *et al*., 1993; Appleton and Vickerman, 1998; Shields and Squyars 2000; Stentiford and Shields, 2005; Li *et al*., 2011). Additionally, the role of cannibalism in *Hematodinium* transmission has been investigated in *C. sapidus* with varying results (Walker *et al*.,

2009; Li *et al*., 2011b). Walker *et al*. (2009) showed 73% of naïve crabs became infected with *Hematodinium* after eating infected tissues, whereas Li *et al*. (2011b) were only able to generate *Hematodinium* infections in 2 of 120 naïve crabs. Reasons for this incongruence could be that some of the crabs used by Walker *et al.* (2009) possessed occult infections, while the crabs in the study of Li *et al*. (2011b) were not exposed to a sufficient amount of the parasite within the infected tissue or for sufficient time. Cannabilism as a mode of transmission is possible, but more research is needed to determine its role, if any, in the spread of infection.

Additionally, larger crabs may be more resistant to the parasite than younger or recently moulted crabs, which would explain the lower prevalence in old hard shelled crabs (Messick and Shields, 2000). Another consideration is that *Hematodinium* may utilize intermediate or reservoir hosts. *Hematodinium* DNA has been detected in association with caprellid amphipods in locations where infected crabs were also detected (Pagenkopp Lohan *et al*., 2012). As the amphipods studied were filter feeders, perhaps *Hematodinium* DNA from the gut contents was amplified; however, the parasite has been detected histologically in several amphipod species (Johnson, 1986), many of which are eaten by other crustaceans. Thus, amphipods may serve as potential reservoir hosts for *Hematodinium*. *Hematodinium* may also utilize other decapods as reservoir hosts. Pagenkopp Lohan *et al*. (2012) found elevated prevalences of *Hematodinium* in several decapod species (spider crabs, *Libinia dubia, L emarginata*, and mud crabs). *Hematodinium* in this study was genetically similar to those found in blue crabs. If these truly represent reservoir hosts, the parasite would be able to survive if populations of other decapod species declined. Nevertheless, little work regarding the transmissibility

of *Hematodinium* through crustaceans has been completed. Further research on potential reservoir hosts and the possible trophic transmission of *Hematodinium* would be beneficial for understanding the parasite's mode of transmission.

In *C. sapidus*, *Ch. opilio and Ch. bairdi,* there is a strong correlation between *Hematodinium* infection and season (Meyers *et al*., 1990; Messick and Shields, 2000; Mullowney *et al*., 2011). In these species, ecdysis occurs during the spring and summer months as water temperatures increase. *Hematodinium* prevalence in these species peak in the fall months and in recently moulted crustaceans, lending support to the hypothesis that *Hematodinium* infections are more likely to occur during ecdysis (Meyers *et al*., 1990; Messick and Shields, 2000; Mullowney *et al*., 2011). To date, no transmission experiments, where crustaceans have been exposed to viable parasites during moulting, have been reported. This is due in part to the difficulty in both obtaining parasites and in housing and keeping crustaceans alive during this stressful period. Evidently, there is a need for further investigation to determine the intricacies of *Hematodinium* infection and disease in crustaceans.

1.6 Prevalence of Hematodinium

Hematodinium parasitizes over 40 crustacean species worldwide (Morado, 2011) many of which have economic significance. *Hematodinium* was first reported in the harbour crab, *Liocarcinus depurator,* and European shore crab, *Carcinus maenas,* off the Normandy and Mediterranean coasts of France in 1931 but were considered extremely rare (<0.1%) and only motile dinospores were observed (Chatton and Poisson, 1931). Subsequently, there have been numerous outbreaks in other crustaceans: edible crab (*Cancer pagurus*), Tanner crab (*Chionoecetes bairdi*), blue crab (*Callinectes sapidus*),

Norway lobster (*Nephrops norvegicus*), and velvet crab (*Necora puber*) (Meyers *et al*., 1987; Latrouite *et al*., 1988; Field *et al*., 1992; Messick, 1994; Messick and Shields 2000; Wilhelm and Miahle, 1996; Stentiford *et al*., 2002). The first confirmed case of *Hematodinium* infection in North American waters was in *C. sapidus* (Newman and Johnson, 1975). *Hematodinium* infections have since been linked with summer die-offs of *C. sapidus* (Sheppard *et al*., 2003) in Wassaw Sound, Georgia, and are a major concern for that fishery. By 1990, the parasite was reported in Newfoundland snow crabs (*Chionoecetes opilio*) (Taylor and Khan, 1995) with seasonal prevalence reaching 20% in some areas, making *Hematodinium* a potential threat to *C. opilio* populations and the lucrative fishery (Mullowney *et al*., 2011). Furthermore, parasitism by *Hematodinium* apparently causes the eventual death of the host (Field *et al*., 1992). Thus, with up to 20% of crabs (consisting mostly of juveniles) being infected and subsequently dying, *Hematodinium* greatly impacts the rate at which snow crab stocks replenish. Combined with the impact of commercial fishing on snow crab stocks, the sustainability of this fishery becomes more difficult.

Hematodinium was found in northeastern Alaska by 1985 and Australia by 1992 (Meyers *et al*., 1987; Hudson and Shields, 1994). The cause of the spread of this disease agent is not well understood. Several dispersal hypotheses have been generated for *Hematodinium*, including transport in ballast water, transport via ocean currents, and transport via infected hosts. The longevity of dinospores, the apparent infectious stage of *Hematodinium,* have been shown to survive up to 73 days in sterile seawater, but their survivability in normal conditions is unknown as cultures were destroyed by bacterial contaminants (Meyers *et al*., 1987). A recent *in vitro* study of *Hematodinium* from blue

crab reported dinospores surviving only seven days in culture (Li *et al*., 2011). As they likely cannot compete well against bacteria, and they may only survive short periods, their dispersal probability is likely limited. The spread of invasive crab species, however, has been documented, including species that are known to be susceptible to *Hematodinium* infection making this scenario likely (Carlton and Cohen 2003).

Few studies have sampled crustacean hosts year round over several years, which limit our knowledge of disease susceptibility, seasonal prevalence, and overall impact of *Hematodinium* on crustacean species. Although this need exists, significant trends and hypotheses have arisen from many of these seasonal surveys. First, for Norway lobster there is a positive correlation between disease caused by *Hematodinium* and salinity (Briggs and McAliskey, 2002). Furthermore, there is an apparent salinity threshold of *Hematodinium* in blue crabs and snow crabs. *Hematodinium* may proliferate within blue crab at low salinities (5ppt), but are incapable of transmission in this environment (Coffey *et al*., 2012). To date, *Hematodinium* has not been observed in snow crab in salinities < 33 ppt (Mullowney *et al*., 2011). Furthermore, recent studies of *C. opilio* infections suggest that disease prevalence is host density dependent (Mullowney *et al*., 2011). Thus, salinity, density, and other factors likely play a role in spatial patterns, periodicity, and transmission of the disease.

Hematodinium has seasonal increases in prevalence, lower in the late summer and fall months and elevated in spring and early summer (Meyers *et al*., 1990; Love *et al*., 1993; Field *et al*., 1998; Messick and Shields 2000; Briggs and McAliskey 2002; Sheppard *et al.*, 2003; Mullowney *et al*., 2011). This seasonality is in part due to the moult cycle (Meyers *et al*., 1990, Stentiford *et al*., 2001). Moulting is very stressful to

crustaceans and typically occurs during the spring although younger, smaller crabs typically moult more often. *Chionoecetes opilio* increase 28-36% in volume and up to 26% in size after moulting (Robichaud *et al*., 1989; Hoenig *et al*., 1994). Susceptible crustaceans presumably acquire the parasite during this time. To fill their new shell, crustaceans take up copious amounts of water from the surrounding environment. Additionally, new shells are soft, making it more susceptible to damage; if damage occurred, opportunistic infections of *Hematodinium* could arise through these portals of entry. This would coincide with increased detection of *Hematodinium* in late summer or fall, and would account for higher prevalence in younger crabs (Mullowney *et al*., 2011).

1.7 Research objectives and rationale

The current study investigates the life cycle of *Hematodinium in vitro*, isolated from Atlantic snow crabs, *C. opilio*. The life cycle of *Hematodinium* isolated from other crustacean hosts has been studied, most extensively in *N. norvegicus* (Appleton and Vickerman, 1998), but also recently from blue crab, *C. sapidus* (Li *et al*., 2011). The principle hypothesis was that similar life stages to those described by Appleton and Vickerman (1998) and Li *et al*. (2011) would be observed in *Hematodinium* isolated and cultured *in vitro* from *C. opilio* hemolymph. The main objectives were to 1) successfully culture the parasite *in vitro*, and 2) characterize all observed life stages using light and electron (scanning and transmission) microscopy. The justification for using *in vitro* methods for the present study were: 1) results would be directly comparable to previously published work on *Hematodinium* isolated from other hosts; 2) *in vitro* studies are more cost effective and less variable than *in vivo* studies; it is easier to manage cultures and alter environmental conditions; and 3) *in vitro* settings enable one to study the parasite

without any of the confounding factors associated with the host. *Hematodinium* from *C. opilio w*ere chosen because 1) there have been no studies investigating the *in vitro* development of *Hematodinium* from this cold water host and 2) the economic significance of this crustacean species to Atlantic Canada.

The secondary hypothesis in the present investigation was that *Hematodinium* isolated from a cold water host would be more amenable to cryopreservation than one from warm water. The objective was to test the suitability of *Hematodinium* isolated from *C. opilio* to be cryopreserved under standard conditions. If successful, this would provide a renewable source of viable *Hematodinium* for future studies by depositing the cells in a readily accessible repository. Knowledge gained in the present study will hopefully give researchers a better understanding of the basic biology of *Hematodinium* and will provide a foundation for future studies involving disease transmission, parasite mitigation, and comprehensive life stage analysis.

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Chapter 2: IN VITRO CULTIVATION OF *HEMATODINIUM* SP. AND DESCRIPTION OF LIFE STAGES USING LIGHT AND ELECTRON MICROSCOPY

2.1 Introduction

Hematodinium perezi was first reported in the European shore crab, *Carcinus maenas* and the harbour crab, *Liocarcinus depurator* in the waters off the Normandy coast of France in 1931 (Chatton and Poisson, 1931). The original morphological description of this parasitic dinoflagellate was illustrated with hand drawings and no isolates were preserved or cultured. *Hematodinium* sp. remained largely unnoticed until the late 1960's, when a morphologically similar parasite was observed in the hemolymph of blue crabs, *Callinectes sapidus* off the coast of North Carolina, Georgia, and Florida (Newman and Johnson, 1975). Subsequently, *Hematodinium* spp. have been identified worldwide, infecting numerous economically significant crustacean species (Chatton and Poisson, 1931; Newman and Johnson, 1975; Maclean and Ruddell, 1978; Meyers *et al*., 1987; Latrouite *et al*., 1988; Wilhelm and Boulo, 1988; Hudson and Lester, 1994; Hudson and Shields, 1994; Messick, 1994; Taylor and Khan, 1995; Wilhelm and Mialhe, 1996, Ryazanova *et al*., 2010).

Hematodinium sp. are also associated with the Atlantic Snow Crab, *Chionoecetes opilio*, an economically important decapod species in North Atlantic and Pacific regions. Approximately 84,000 metric tons of *C. opilio* were harvested in 2010 in the North Atlantic region, and was valued at over $459 million CAD (http://www.dfo-mpo.gc.ca/stats/commercial/land-debarq/sea-maritimes/s2011av-eng.htm -accessed June 14, 2013). In 1990, *Hematodinium* was first reported in *C. opilio* off the coast of Newfoundland and Labrador, and was observed with relatively low prevalence (<0.11% - 3.7%) in the years following (Taylor and Khan, 1995). Subsequently, peaks in

Hematodinium prevalence have approached 20% in some areas, with increased prevalences occurring in juvenile and female crabs (Mullowney *et al*., 2011). Furthermore, once a crab acquires infection, the prevailing view is that the crab will eventually die and therefore, the parasite may have considerable impact on populations of wild crustacean stocks in the region, ultimately affecting the economic vigour of coastal maritime communities.

During early stages of infection, *Hematodinium* is seen within hemal spaces associated with the hepatopancreas (Field and Appleton 1996, Appleton and Vickerman 1998, Stentiford *et al*., 2001b). As infections progress, the parasite circulates in hemolymph and is seen in gills, heart, skeletal muscle, gut, antennal gland, eyestalk, and brain (Newman and Johnson, 1975; MacLean and Ruddell, 1978; Meyers *et al*., 1987; Field *et al*., 1992; Love *et al*., 1993; Hudson and Shields, 1994; Messick, 1994; Shields, 1994; Field and Appleton, 1995; Taylor and Khan, 1995; Taylor *et al*., 1996; Wilhelm and Mialhe, 1996; Shields and Squyars, 2000; Stentiford *et al*., 2002; Ryazanova *et al*., 2010; Small *et al*., 2012). Parasites are attached to the epithelium lining hemolymph-containing vessels but not within organ parenchyma (Field and Appleton, 1995; Small *et al.*, 2012). During late stages of infection, the host experiences hemocytopenia that leads to respiratory and organ malfunctions which may ultimately contribute to the host's death (Shields, 1994; Field and Appleton, 1995, Shields and Squyars, 2000).

Although *Hematodinium* has been observed for over 80 years and has had major impacts on multiple crustacean fisheries, many aspects of the parasite's biology remain elusive. A lack of an *in vitro* culture system for *Hematodinium* has limited our understanding of the parasite's life cycle stages.

Gross pathological changes in the host are associated with late stage infections; these include cuticle discolouration and milky white hemolymph associated with large numbers of parasites (Stentiford and Shields, 2005). Furthermore, direct correlation exists between the crustacean host's moult cycle and *Hematodinium* seasonal cycle or disease progression within the host (Eaton *et al*., 1991; Stentiford *et al*., 2001a, Stentiford and Shields, 2005; Mullowney *et al*., 2011). *Hematodinium* is observed more commonly in juvenile crustaceans as they moult more frequently and this, associated with anecdotal evidence that all infected crabs succumb to death, may affect recruitment (Messick, 1994; Wilhelm and Mialhe, 1996; Dawe *et al*., 2001, 2002; Stentiford *et al*., 2001a; Shields *et al*., 2005). As increased prevalence is associated with small crustaceans, which are not caught using conventional techniques, the ability to obtain infected specimens and subsequent parasites remains difficult. Furthermore, isolating *Hematodinium* from *C. opilio* becomes even harder as the crab lives in deep water (100-400 m). Efforts to sample crustaceans from areas with increased *Hematodinium* prevalences/outbreaks and proper specimen storage and transport may improve parasite survival and aid in the investigation of many facets of the parasite's physiology, biochemistry and genetics, epidemiology, transmission, pathogenesis, cell biology and environmental tolerances (Hutchison, 1976; Dubey, 2009; Roche and Guégan, 2011; Stentiford and Neil, 2011). Without proper knowledge of a parasite's life history, mitigating disease and predicting environmental impacts is also difficult (WHO, 2008; Stentiford *et al*., 2012).

Hematodinium's life cycle can be differentiated into three life stages, which are generally agreed upon, based on natural infections: trophonts, sporonts, and dinospores (Meyers *et al*., 1987; Eaton *et al*., 1991; Stentiford and Shields, 2005). Trophonts

measure 12-58 μm in length and are the vegetative form of the parasite (Appleton and Vickerman, 1998; Li *et al*., 2011). They have been described *in vivo* as forming 'sheets' near various organs, especially the hepatopancreas (Field and Appleton, 1995; Wheeler *et al*., 2007; Chualáin and Robinson, 2011). Trophonts appear vacuolated or 'foamy' when viewed by light microscopy and can be uninucleated or multinucleated (Eaton *et al*., 1991; Appleton and Vickerman, 1998; Morado, 2007; Ryazanova, 2008; Li *et al*., 2011). Trophonts give rise to sporonts, which have also been described as sporoblasts and pre-spores (Appleton and Vickerman, 1998; Stentiford and Shields, 2005; Ryazanova *et al*., 2008; Li *et al*., 2011). A distinction between sporonts and pre-spores has been made by some authors, highlighting slight variation in cell size; however, this appears subjective and is not consistently applied (Appleton and Vickerman, 1998; Li *et al*., 2011). A common distinction between trophont and sporont stages is the appearance of trichocysts in sporont stages (Meyers *et al*., 1987; Appleton and Vickerman, 1998; Li *et al*., 2011). Trichocysts are extrusomal organelles that are long, rectangular, and electron dense when viewed ultrastructurally. Sporonts give rise to dinospores, which are the motile, dispersal form of the parasite (Appleton and Vickerman, 1998; Stentiford and Shields, 2005; Li *et al*., 2011). Each dinospore has two flagella, one transverse and one longitudinal, which propel and direct the parasite respectively. Two types of *Hematodinium* dinospores, macrodinospores and microdinospores, occur in several crustacean hosts. Macrodinospores are ellipsoid and measure 16-20 μm long and 8.5-11.5 μm wide. Microdinospores are smaller, measuring 11-14 μm long and 4.5-6.5 μm wide, and have a 'corkscrew' shape (Appleton and Vickerman, 1998). Additionally, *Hematodinium* infections from a single crab will give rise to only one type of dinospore (Meyers *et al*.,

1987; Appleton and Vickerman, 1998; Li *et al*., 2011). Although one might speculate that dinospores represent anisogamous gametes, Appleton and Vickerman (1998) showed that a single dimorphism can generate amoeboid trophonts, thus rendering that scenario unlikely. Dinospores exit the host through the mouth and gills in 'plumes', sometimes at very high densities (1.6×10^8 cell ml^{-1}) (Stentiford and Shields, 2005). Collectively, this evidence supports a possible role in transmission for dinospores *in vivo,* although the evolutionary significance of dinospore dimorphisms in *Hematodinium* remains unclear.

Surprisingly, there have only been two studies investigating *Hematodinium* sp. *in vitro*. Appleton and Vickerman (1998) first isolated *Hematodinium* from the Norway lobster, *Nephrops norvegicus* and more recently, Li *et al*., (2011) isolated *Hematodinium* from the blue crab, *C. sapidus*. Both studies identified life stages and presented a life history using light microscopy but only Appleton and Vickerman (1998) used electron microscopy in their analysis. No *in vitro* study of *Hematodinium* isolated from snow crabs, *C. opilio* has been completed. *Chionoecetes opilio* prefer temperatures below 3°C (Tremblay, 1997), which is much colder than either *N. norvegicus* or *C. sapidus*. Optimal temperature ranges for *C. sapidus* and *N. norvegicus* are 15-30°C and 4-16°C respectively but may vary depending on salinity (Cadman and Weinstein, 1988; Baden *et al*., 1990; Bateman *et al*., 2012). Thus, an investigation was initiated to study *Hematodinium in vitro* and to characterize life stages using both light and electron microscopy. *Hematodinium* isolated from *C. opilio* was used because of its negative impact to local fisheries in the area (the parasite has been described as a regional concern by the DFO-MPO). In an area that has already suffered the collapse of a major cod fishery (Harris, 1998), *Hematodinium* may affect population stocks of the North Atlantic

snow crab fishery, exacerbating the difficult economic conditions of the area. Having *in vitro* cultures of the parasite will facilitate a better understanding of the individual life stages, the environmental conditions that best support growth, replication and the transitions between stages. Ultimately, this may lead to better characterization of the parasite's life stages *in vivo* and toward understanding environmental tolerances of the parasite. This is the first investigation of *in vitro* life stages of *Hematodinium* isolated from *C. opilio,* and is only the second investigation using ultrastructural analyses to investigate life stages of *Hematodinium*.

2.2 Materials and Methods

For the purpose of this thesis, methods will be divided into the year in which they were performed, as adaptations were made in 2011 based on the results of 2010.

2.2.1 Year 1 (2010)

2.2.1.1 Collection and shipment of C. opilio

Thirteen crabs showing morphological changes (discoloured or opaque cuticles and milky white hemolymph) consistent with Bitter Crab Disease (BCD) (12 from Newfoundland, one from Nova Scotia) were obtained.

Atlantic snow crabs (*C. opilio*) were collected during annual surveys in Notre Dame Bay, Newfoundland during September 2010. Crabs were caught by pot trap on the grounds of strata 610 and 611 (200-400 m in depth). Water temperatures for strata 610 and 611 range between -1°C and 3°C in early fall (Mullowney *et al*., 2011). Crabs were kept in coolers layered between seawater soaked burlap, and placed above saltwater ice until reaching shore. On shore, coolers were transported to St. John's, Newfoundland, where they were sent via air cargo to the Atlantic Veterinary College (AVC), University

of Prince Edward. The interval between snow crab harvest and their arrival at the AVC was usually 24-48 h. During the post-season survey (mid-late October), hemolymph was drawn aseptically from a total of 72 suspected *Hematodinium* infected snow crabs on board the vessel using either 1 ml or 3 ml syringes. These were kept on ice and also shipped via air cargo to the AVC, University of Prince Edward Island until transferred to culture medium.

One additional crab was obtained off the coast of Nova Scotia at position 44° 34.86N, 58° 40.29W during October, 2010. Water depth was ~100 m and bottom temperatures typically range between 0-3°C at this location (Tremblay, 1997). This crab was kept in a cooler packed with salt water ice and transported by land to the AVC. The interval between snow crab harvest and its arrival at the AVC was approximately 72 h.

2.2.1.2 Necropsy of *C. opilio and hemolymph collection*

Upon arrival, crabs were stored in an aerated holding tank at 0°C with 33-34 ppt salinity for 1-4 h to recover. Ice packs were placed in the holding tank in an attempt to keep water temperatures at 0°C. Crab viability was assessed by observing mandible or limb movement and response to 70% ethanol (EtOH) sprayed on the mouth and gills. Only live crabs were used in the isolation of dinoflagellates. After carapace width, sex, hemolymph refractive index, and any gross lesions were recorded, crabs were exsanguinated. Hemolymph was collected aseptically from the base of the first periopod using a 3 ml or 5 ml syringe with 22 gauge needle. Hemolymph (200 µl) was placed into a 96 well plate containing 800 µl 80% ethanol for DNA extraction and genotype confirmation by polymerase chain reaction (as part of a separate study), and one drop (~15 µl) was smeared onto a glass microscope slide. Air-dried smears were later stained

with Wright-Giemsa in a Hema-Tek 1000 automated slide stainer (Miles Scientific). Remaining hemolymph was stored on ice until completion of all necropsies. After exsanguination, the carapace of the crab was removed, and the heart, hepatopancreas, eyestalk, gills, gonad, gut, and leg muscle were surgically removed and placed in Davidson's seawater fixative for future histopathological examination (as part of Dr. Melanie Buote's Ph.D. research). Necropsies usually took 3-4 h, depending on the number of crabs obtained in each shipment.

2.2.1.3 Initiation and maintenance of cultures

After all necropsies were completed, hemolymph containing suspect *Hematodinium* from BCD evident crabs was cultured *in vitro*. Syringes containing hemolymph were gently agitated to ensure adequate mixing, and hemolymph was transferred to 1.7 ml microcentrifuge tubes within a Class II biological safety cabinet (Sterigard® III Advance, The Baker Company). Infected hemolymph (40 μl) was added to 1.94 ml of each of the following media in 24 multiwell plates (Falcon) and incubated in at 0 ± 0.2°C: *Nephrops* medium (See Appendix A), Modified Ciliate medium (See Appendix B), and Dulbecco's Modified Eagles Medium (DMEM):Hams F12 (See Appendix C). Cultures were viewed on an inverted phase contrast microscope (Nikon) with transmitted light every two to three days to monitor growth and the possible presence of contaminating organisms. If cultures became too dense (cells covered ~2/3 of culture well), they were diluted 1:1 with fresh medium. If cultures developed contaminating organisms, a 1 ml aliquot of sample was sent to AVC Diagnostic Services, where a series of tests were performed to classify the organism and elucidate effective

treatment options. If contamination could not be resolved quickly, the culture was discarded.

Culture medium was changed every 7-10 days using the following procedure: gentle pipetting of cultures were completed to resuspend any non-adherent cells and a 1 ml aliquot was transferred into microcentrifuge tubes, and centrifuged at 900 X g for 5 min at 4°C; care was taken to minimize disruption of cells that were attached to plate surface. Supernatant was discarded, and the cell pellet was resuspended in 1 ml of fresh medium. The fresh suspension was transferred gently by pipette into its original culture well, which had been kept at 0 ± 0.2°C during centrifugation.

2.2.1.4 Preparation of samples for vital staining

Samples were periodically assessed for viability by staining with 0.2% Neutral Red (Sigma-Aldrich) or 0.05% Acridine Orange (Sigma-Aldrich). One drop of sample (~15µl) was placed on a glass microscope slide within a Class II biological safety cabinet (Sterigard® III Advance, The Baker Company), mixed with an equal volume of doubly concentrated vital stain, and viewed immediately on an Axioplan 2 imaging microscope (Zeiss). Samples stained with Neutral Red were viewed with transmitted light, and those stained with Acridine Orange were viewed with ultraviolet light passed through a FITC filter (Zeiss). All images were captured using an Axiocam ACC camera.

2.2.1.5 Preparation and imaging of samples for scanning electron microscopy

Hematodinium, from cultures initiated in the fall of 2010, were processed in March, 2011, for scanning electron microscopy (SEM). A 1 ml aliquot of culture was fixed in an equal volume of 6% glutaraldehyde in artificial sea water (ASW; Instant Ocean) (33ppt). After solutions were mixed, they were immediately drawn by syringe

and pressed onto a 0.2 μm nitrocellulose filter (Millipore) within a filter holder (Millipore) contained in a glass beaker. Artificial sea water was added up to the level of the filter to prevent sample desiccation and incubated overnight at 4°C. After incubation, the filter was gently washed 2X with ASW for 2 min, and post-fixed with 1% OsO_4 in ASW for 2 min. Samples were dehydrated using a graded series of ethanol (EtOH) washes at concentrations of 20%, 35%, 50%, and 70%. Each wash was performed 2X and was 2 min in duration. The filter holder apparatus in 70% EtOH was sent to Jim Ehrman, at the Digital Microscope Facility in Mount Allison University, Sackville, NB. Mr. Ehrman continued to dehydrate the samples using a graded series of EtOH concentrations of 85%, 95% and 100%. Each wash was performed 1X for 10 min with the exception of the last wash, which was performed 4X. Samples were critically point dried with liquid CO_2 using a Denton DCP-1 critical point dryer (Denton Vacuum). Samples were mounted on filters on 10 mm X 10 mm aluminum stubs (Electron Microscopy Sciences) using double sided tape. The filters were rimmed with colloidal graphite (Electron Microscopic Sciences) and coated with ~15 nm gold using a Hummer 6.2 sputtering unit (Anatech Ltd.). Specimens were examined using a JEOL JSM-5600 scanning electron microscope operating at 10 kV and 8 mm working distance. Images were acquired using the built in digital image capture device on the scanning electron microscope.

2.2.1.6 Preparation and imaging of samples for transmission electron microscopy

Hemolymph from BCD suspect crabs was fixed for transmission electron microscopy (TEM) the same day as necropsies were conducted. Hemolymph (~75 μl) was added to 2 ml of 3% glutaraldehyde (Canemco) in ASW and stored at 0°C for either 12 days (Newfoundland sample) or 24 h (Nova Scotia sample). Samples from

Newfoundland were kept in fixative longer as part of a course project assessing tissue quality after different fixation periods. Samples were washed twice in ASW and post-fixed in 1% OsO_4 in ASW for 30 min. Prior to washing, samples were centrifuged at 1700 X g at room temperature (RT; 20-22°C) for 5 min. During washing, samples were centrifuged at 3800 X g at RT for 10 min. After post-fixation, samples were centrifuged at 6700 X g for 10 min at RT. Osmium tetroxide was removed from above the pelleted samples, and the pellets were overlaid with 4% aqueous molten agar (Fisher). Samples were incubated at 4°C for 10 min to solidify the agar. Samples were removed from the microcentrifuge tube, cut into small pieces with razor blades, and placed in glass vials containing 50% EtOH. Samples were dehydrated using a graded series of EtOH washes at concentrations of 50%, 70%, 95%, and 100%, then cleared in propylene oxide (PO). Each wash was performed 2X for 10 min each with the exception of the first step, where only one wash was performed. After clearing in PO, samples were infiltrated with Epon resin by placing them in a mixture of Epon/PO at a ratio of 1:1 for 1 h, at a ratio of 3:1 for 2 h, and then in 100% Epon overnight in vacuum dessicator at RT. Epon resin was prepared as described in Appendix D. After infiltration, samples were placed in BEEM capsules (Canemco) filled with Epon, and incubated at 60°C overnight to polymerize the Epon.

Cultures from 2010 crabs were fixed as follows: one millilitre of 6% glutaraldehyde in *Nephrops* saline at pH 7.6 was added to equal amounts of culture, mixed thoroughly by pipetting, and incubated overnight at 4°C. *Nephrops* saline differs from *Nephrop*s medium in that it does not contain antibiotics or foetal bovine serum. Samples were washed, post-fixed, overlaid in agar, dehydrated, infiltrated with Epon and

polymerized as described above with the exception of centrifugation being performed at 1700 X g for washes as increased centrifugation was not needed to obtain pellets.

Thick sections (0.5 μm) were cut with glass knives (Leica) on an ultramicrotome (Reichert-Jung, Ultracut E, Leica Microsystems), mounted on glass slides, and stained with 1% toluidine blue in 1% sodium tetraborate solution and examined with a light microscope to select areas for ultrathin sectioning.

Ultrathin sections (90 nm) were cut with glass knives on an ultramicrotome. Sections were retrieved onto 200 mesh copper SuperGrids™ (SPI Supplies) and stained with uranyl acetate (saturated aqueous solution) for 30 min at RT, then stained with lead acetate for 2 min at RT, as described elsewhere (Sato, 1968). After each staining period, samples were triple washed in dH_2O for 30 sec. Sections were viewed with a Hitachi H7500 transmission electron microscope operated at 80 kV and digital images were taken using an AMP XR40 side mounted camera.

2.2.2 Year 2(2011)

2.2.2.1 Collection and shipment of C. opilio

Atlantic snow crabs (*C. opilio*) were collected during annual surveys in White Bay, Newfoundland in September 2011. Crabs were caught by pot trap on the grounds of stratum 614 (300-401 m in depth). The water temperatures for stratum 614 normally ranged between 0-1°C in early fall (Mullowney *et al*., 2011). Crabs were maintained aboard ship and shipped post-harvest as described above. Eight crabs showed morphological changes consistent with BCD. One additional crab was obtained off the coast of Nova Scotia at an approximate position of 44° 23.23N, 63° 28.11W during the same period. The water depth was ~100 m and bottom temperature typically ranges

between 0-3°C at this location (Tremblay, 1997). Upon arrival, crabs were examined at necropsy and hemolymph collected as described above.

2.2.2.2 Initiation and maintenance of cell cultures

After necropsy, hemolymph containing suspect *Hematodinium* from BCD evident crabs was cultured *in vitro*. Syringes containing hemolymph were gently agitated as described above. *Hematodinium* was cultured in *Nephrops* medium solely, based on results from the previous year. Initiation and maintenance of culture was performed as described above; however, one culture replicate from each sample was incubated at 4°C to investigate the effect of increased temperatures. Samples #64, #98 were split three times within the first two weeks of culture; Samples #62, #65, and NS-1 were split once per week for the first two weeks of culture. Only Samples #65 and NS-1 had further splitting, which occurred during late-stage sporont development. Cultures were terminated after sixteen weeks *in vitro*.

2.2.2.3 Preparation of samples for vital staining

Samples were periodically assessed for viability by staining with 0.2% Neutral Red (Sigma-Aldrich) or 0.05% Acridine Orange (Sigma-Aldrich). Samples were stained 24 h after incubation, 3 days following, and after any notable changes in cell morphology, which was often every week for the first month of culturing. Staining and imaging of specimens was performed as described above.

2.2.2.4 Preparation and imaging of samples for scanning electron microscopy

Initial hemolymph samples were fixed immediately after necropsies for SEM. Infected hemolymph (50 µl) was fixed with 1 ml of 3% glutaraldehyde in ASW in a 2 ml culture well. The solution was immediately drawn by syringe and pressed onto a 0.2 µm

polycarbonate filter (Sterlitech) contained in a filter holder (Millipore) in a glass beaker. Artificial sea water was added up to the level of the filter and incubated overnight at 4°C. The sample was washed gently with ASW, post-fixed in OsO_4, and dehydrated as described above. The filter was gently removed from the filter holder, placed between two galvanized washers, and clamped using metal binder clamps. This assembly was placed in a beaker containing 70% EtOH and shipped to Jim Ehrman. Mr. Ehrman continued dehydration, dried, mounted, and imaged samples as described above.

In addition to initial hemolymph collections, the *in vitro* culture was processed for and viewed using scanning electron microscopy. Samples were fixed and processed either one or two weeks following initial hemolymph collection, and every two weeks subsequently. In some instances, samples were not fixed at a scheduled time point due to either insufficient sample volume or gross contamination, which would have affected image quality. Samples were fixed by adding 1 ml of 6% glutaraldehyde (Canemco) to the contents of a culture well (~2ml) and mixed thoroughly by pipette. The solution was pressed onto a 0.2 µm polycarbonate filter, processed, and imaged as described above, with the exception that samples were washed with *Nephrops* saline instead of ASW. After 2-3 sampling time points, it was noted that the fixation quality was not optimal. The fixation and processing procedure was then modified as follows: cultures were initially fixed by adding 1 ml of 1% OsO_4 in ASW to culture wells. These were then mixed thoroughly by pipette, drawn by syringe and pressed onto a 0.2 µm polycarbonate filter as above. *Nephrops* saline was added to the level of the filter, and the sample was incubated for 2 min at RT. It was then rinsed gently with *Nephrops* saline 2X and post-fixed with 3% glutaraldehyde, and incubated at 0°C overnight. After incubation, the

sample was washed 2X with *Nephrops* saline and 1X with 20% EtOH. The filter was removed gently and stacked between galvanized washers as described above, and placed in Petri dishes. The samples were washed additionally with 20% EtOH and subsequently with 35%, 50%, and 70% EtOH as above. The filter containing apparatus was then placed in a container filled with 70% EtOH and shipped to Jim Ehrman at Mount Allison University, where the samples were dehydrated, dried, mounted, coated, and imaged as described above.

2.2.2.5 Preparation and imaging of samples for TEM

Hemolymph (150 µl) from BCD suspect crabs was fixed the same day as necropsies were conducted. In addition, samples from *in vitro* culture were fixed and processed either one or two weeks following initial hemolymph collection and every two weeks following, up to sixteen weeks. Some cultures however, were not fixed at their appointed time points due to bacterial contamination or lack of sample volume, which would have affected image quality and results. Hemolymph was fixed in 3% glutaraldehyde immediately following necropsies, incubated overnight at 0°C and further processed as described for 2010 cultures. 2011 cultures were fixed and processed similar to 2010 cultures. Cultures were sectioned and viewed as described for 2010 samples except that ultrathin sections were cut using a diamond knife (Pelco).

2.2.2.6 Measurements and statistics

Hematodinium cell dimensions were measured using FIJI (FIJI is a freeware program based on Image J and can be downloaded at http://fiji.sc). The diameter of 100 lipofuscin-like granules were measured from a representative cell from hemolymph of

crab #99 and another 100 measured from a representative cell from *in vitro* culture derived from the same hemolymph. Descriptive statistics (mean and standard error) and two sample student's t tests at a 0.05 confidence level were performed with Minitab 15 Statistical Software.

2.3 Results

2.3.1 Year 1 (2010)

2.3.1.1 Cultures

Three different media were assessed for *in vitro* cultivation of *Hematodinium*. The DMEM: Ham's F12 did not support *Hematodinium*. After several days in culture there were no viable cells, thus cultures in that medium were discarded. Cells in the modified ciliate medium remained viable for 2-3 months; however, growth or proliferation was not observed and they eventually died. *Nephrops* medium gave favourable results; cells remained viable in this medium, and progression from sporont to schizont forms was observed; therefore, only *Nephrops* medium was used for *in vitro* culture the following year.

2.3.1.2 Sporonts

Hematodinium isolated from infected *C. opilio* hemolymph was in the sporont stage (Figure 2.1). Sporonts, for the purpose of this study, are synonymous with sporoblasts and pre-spores as previously reported (Meyers *et al*., 1987; Eaton *et al*., 1991; Appleton and Vickerman, 1998; Stentiford and Shields, 2005; Morado, 2007; Ryazanova *et al*., 2010; Li *et al*., 2011). They have typical dinoflagellate characteristics including permanently condensed chromosomes, amphiesmal vesicle membranes. Sporonts also possess lipid droplets, mitochondria with tubular cristae, and lipofuscin-like granules, as

is common for all *Hematodinium* life stages. Characteristic of sporont stages is the appearance of extrusion organelles called trichocysts (Figure 2.1). Multiple

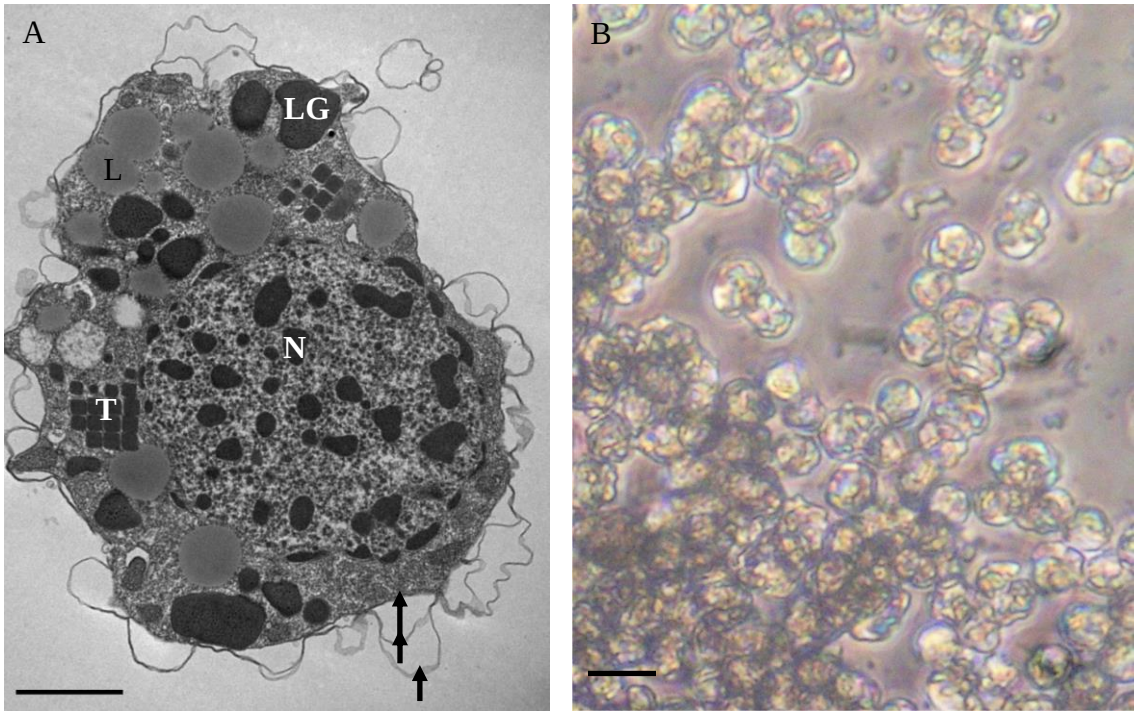

Figure 2.1: A typical sporont from the hemolymph of *C. opilio*. A) A sporont viewed under transmission electron microscopy. The nucleus (N) had permanently beaded chromatin, and the inner and outer amphiesmal vesicle membranes (double arrow) were surrounded by the plasma membrane (arrow). Lipid droplets (L) and lipofuscin-like granules (LG) were also evident. Trichocysts (T), which were characteristic of sporonts, were seen in cross section. Scale bar: 2 µm. B) Individual sporonts within a dense aggregate of similar cells were irregular in morphology when viewed with light microscopy. Organelles specific to sporonts are indistinguishable when viewed by light microscopy. Scale bar: 10 µm.

developmental stages of trichocysts were observed and are described further in Chapter 3.0.

2.3.1.3 Schizont

Schizont forms arose after ~three months *in vitro*. Characteristic of schizonts was their large size (some schizonts exceeded 60 μm in diameter), and a large, relatively empty vesicle with clumps of electron dense granular material, often along the periphery (Figure 2.2). This material was similar to the lipofuscin-like granular material observed in sporonts. These stages were observed in cultures that had been sustained for long periods (>3 months), and were also cultured in suboptimal medium. They were also concurrent with yeast contamination.

2.3.1.4 Contamination issues

Small (4-6 μm long) ellipsoid biflagellate cells developed approximately three months after culturing (Figure 2.2; Figure 2.3). Although their size was smaller than the reported size of microdinospores (11-14 μm, Appleton and Vickerman, 1998), these were initially thought to be *Hematodinium* dinospores, as they were biflagellated and motile.

Following ultrastructural investigations using transmission electron microscopy, these organisms were rejected as *Hematodinium*. They did not have permanently condensed chromosomes typical of dinoflagellates, nor did they have amphiesmal vesicle membranes (Figure 2.4). Furthermore, some developed to a cyst stage which had not been described for *Hematodinium*. These organisms were likely a zoosporic fungi belonging to either Labyrinthulale or Thraustochytriale orders, similar to those reported

by Moss (1985) and Goldstein *et al*., (1964). After these results were obtained, DNA from several cultures was extracted for future PCR genotyping, and all cultures were

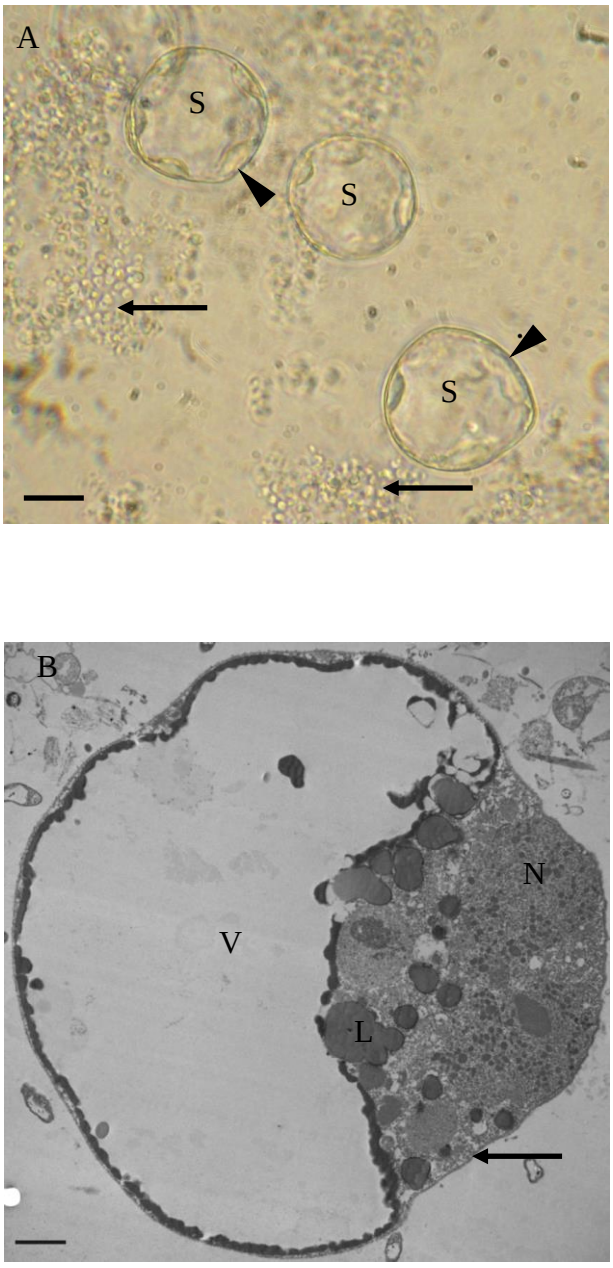

Figure 2.2: Schizonts from 2010 *in vitro* culture A) Several schizonts (S) observed by light microscopy adjacent to numerous small, biflagellated contaminants (arrow). Schizonts appeared as ring shaped due to a large central vesicle. Areas where nuclei and other organelles are situated form bulging regions (arrowhead). Scale bar: 10 µm. B) A binucleated schizont as observed by transmission electron microscopy. The periphery of

the large, relatively empty vacuole (V) was lined with granulated material. Notably, the outer amphiesmal vesicle membrane and plasma membrane (arrow) did not bulge but tightly surrounded the cell. N=nucleus; L= lipid droplet. Scale bar: 2 µm.

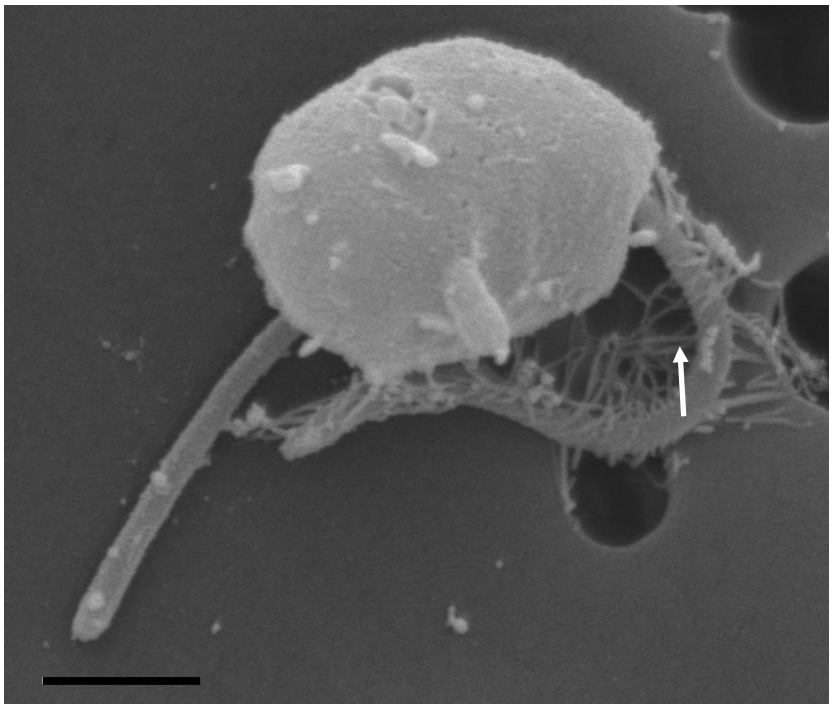

Figure 2.3: Scanning electron micrograph of a biflagellated zoosporic fungal contaminant measuring 4-6 µm from 2010 *in vitro* cultures. Notably, flagellar hairs (arrow) were observed on a flagellum. Scale bar: 2 µm.

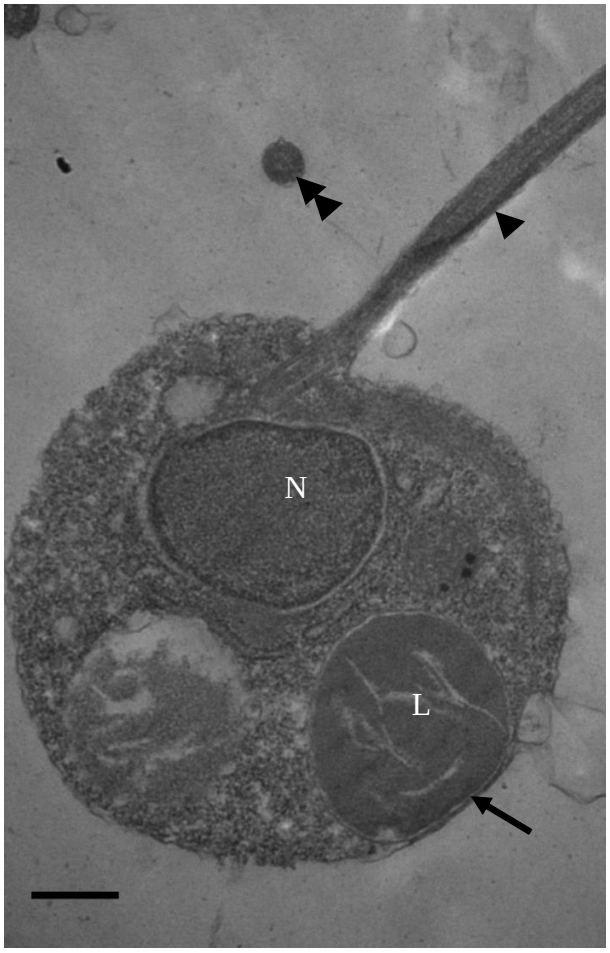

Figure 2.4: Transmission electron micrograph of a biflagellated zoosporic fungus contaminant found within 2010 *in vitro* culture. Two flagella were observed in this image, one cut longitudinal (arrowhead) and one in cross section (double arrowhead). The organism did not have beaded chromatin present in the nucleus (N) or amphiesmal vesicle membranes; a single membrane encapsulated the cell (arrow). L=lipid droplet. Scale bar: 500 nm.

discarded. A PCR assay for the detection of *Hematodinium* was unavailable until spring 2011. After the assay was developed, the fixed samples were processed by Sarah Daley, and confirmed negative for *Hematodinium*.

2.3.2 Year 2 (2011)

A total of six life stages and one senescent stage were observed *in vitro*. A summary of the stages with their corresponding time of fixation for TEM is described in Table 2.1. All life stages had permanently condensed chromosomes, amphiesmal vesicle membranes, lipid droplets and lipofuscin-like granules of varying size. Mitochondria always had tubular cristae, and Golgi often had dilated cisternae.

2.3.2.1 Amoeboid trophonts

From initial hemolymph collection, six of eight samples contained stages similar to amoeboid trophonts described by Appleton and Vickerman (1998). These varied significantly in their morphology but measured ~15-30 µm; some were relatively spherical, while others were elongated or irregular in shape (Figure 2.5). Since they varied significantly in size and morphology, no systematic approach to sizing these stages was undertaken. They were often filled with lipid droplets and lipofuscin-like granules, with little else in the cytoplasm. Furthermore, uninucleated and multinucleated forms were observed. One of the characteristics used in defining trophont stages was their lack of trichocysts. Usually trophonts remained in that life stage for the duration of the sixteen week trial. Occasionally cells within the sample had developing or mature trichocysts when examined ultrastructurally. Trichocysts were not observed from any time points thereafter, indicating that transition only occurred in a small subset of the sample, and did not represent the culture as a whole. Occasionally, singular amoeboid

Table 2.1: A chronology of observed *Hematodinium* life stages corresponding with TEM fixation dates from 2011 samples. Sample names correspond to the crab from which the parasites were isolated. Six life stages were observed, amoeboid trophonts were seen in most initial hemolymph collections with the exception of samples #65 and NS-2011. Cultures in which amoeboid trophonts were found in initial culture often remained at that stage until the completion of the trial or until cultures were terminated. In some cases (#61, #62, #64) early sporonts were observed, but only for one time point. Cultures in which sporonts were observed in initial hemolymph later gave rise to dinospore and post-dinospore stages. Trophont and sporont coenocytes and schizont stages were also observed. Clumps, similar to those described by Appleton and Vickerman (1998) were also observed, but were deemed senescent based on ultrastructural analyses. Some cultures were eventually terminated due to bacterial and yeast contamination. T=Amoeboid trophont; S=Sporont; D= Macrodinospore; PD=Post-dinospore; Sch=Schizont; C=Coenocyte; I.H. =Initial Hemolymph; SC=Senescent clump X= Cultures were terminated.

	2011 Samples							
Date	**#61**	**#62**	**#63**	**#64**	**#65**	**#98**	**#99**	**NS-2011**
5-Sep	I.H.-T	I.H.-T	I.H.-T	I.H.-T	I.H.-S	I.H.-T	I.H.-T	
12-Sep	T			T; Early S		T; C(T)		
19-Sep		T	T		S; C(S)			
23-Sep								I.H.-S
26-Sep	T; Early S		T; SC	T			T	
4-Oct		T	T		S; D			S
11-Oct	T	T; SC					T; C (T)	
17-Oct			T	T	S; D			S
24-Oct	T	T; Early S; Sch (T)				SC		
31-Oct			T; Sch (T)	X	PD			S
7-Nov		T; Sch (S); Sch (T)		X		X		
15-Nov	T			X	X	X	X	
21-Nov			T; Sch (T)	X	X	X	X	S; D
28-Nov		Sch (T)	X	X	X	X	X	
6-Dec			X	X	X	X	X	S; PD; Sch (S)
12-Dec	T	Sch (T)	X	X	X	X	X	
26-Dec	T		X	X	X	X	X	

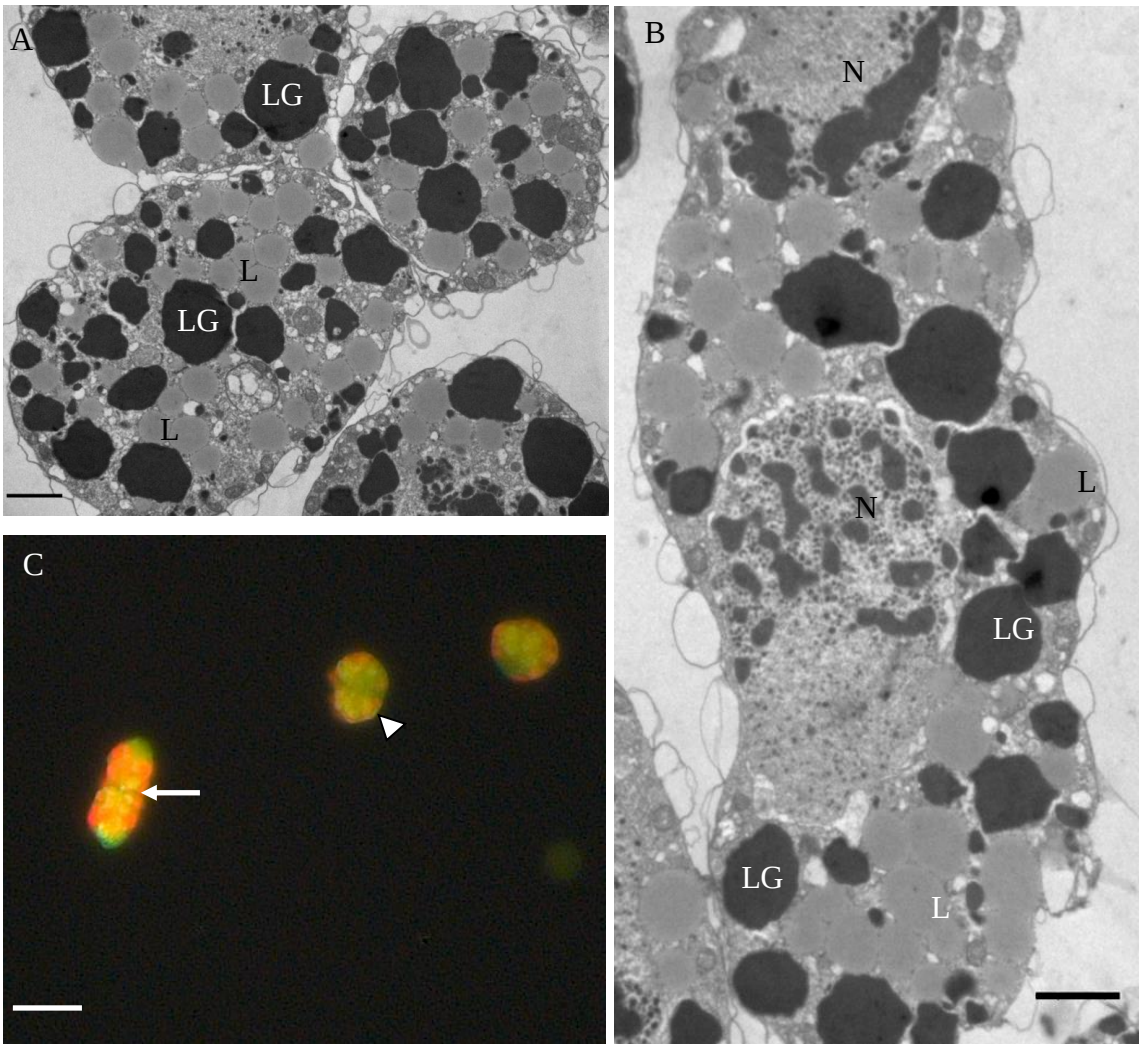

Figure 2.5: Amoeboid trophonts from hemolymph of *C. opilio* caught in 2011. A) Amoeboid trophonts show ovoid or spherical morphology. Much of the cell volume consisted of lipid droplets (L) and electron dense lipofuscin-like granules (LG) and there were no trichocysts present. Scale bar: 2 µm. B) A bi-nucleated amoeboid trophont had filamentous morphology. Although morphology differed from 'A', ultrastructurally they were similar. Lipid droplets (L) and electron dense granules (LG) filled most of the cell and it also lacked trichocysts. Scale bar: 2 µm. C) Amoeboid trophonts with filamentous (arrow) and ovoid morphology (arrowhead) were found within the same cell culture. Cells were stained with Acridine Orange and viewed using fluorescence microscopy. Scale bar: 15 µm. N= nucleus.

trophonts were seen adjacent to large multinucleated masses termed coenocytes.

2.3.2.2 Coenocytes

Coenocytes were observed concurrent with amoeboid trophonts and sporonts. Two types of coenocytes were observed: sheet-like and arachnoid (Figure 2.6; Figure 2.7). They were elaborate networks of interconnected cellular bodies. Both types were observed in association with sporonts; only the sheet-like form was observed in association with amoeboid trophonts. The arachnoid coenocyte attached to the culture well and was very sensitive to mechanical disruption. Changing the medium would often disrupt these networks and only in a few instances did the networks reform. Extensions from arachnoid coenocytes differed from the body of the cell; there were no lipid droplets or lipofuscin-like granules. Furthermore, microfilaments or microtubules were not observed in association with these cytoplasmic extensions (Figure 2.8). It was presumed that the multi-nucleated masses arose from nuclear division without subsequent cytokinesis, since they were associated with dividing cells; therefore, they were termed coenocytes rather than syncytia (Baluška *et al*., 2004). Multinucleated and uninucleated cells appeared to bud from the sheet-like and arachnoid coenocytes (Figure 2.6), likely the result of budding or mechanical disruption to networks.

2.3.2.3 Sporonts

Sporonts, similar to those observed from hemolymph isolates of 2010, were observed from initial hemolymph of two samples (#65 and NS-2011). Sporonts were similar to trophonts ultrastructurally except that they possessed trichocysts (Figure 2.2). Furthermore, there appeared to be variation of sporonts over time. In recently extracted hemolymph, and shortly thereafter, sporonts were irregular in shape, similar to trophont

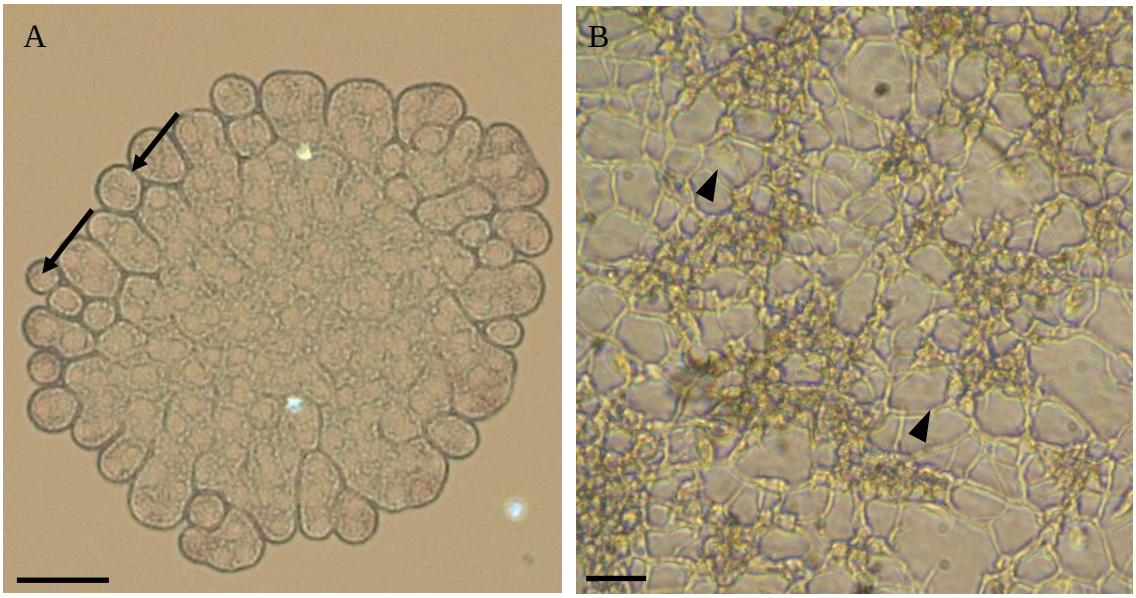

Figure 2.6: Coenocytes from 2011 *in vitro* cultures seen by light microscopy. A) A multi-nucleated central mass with uni- and multinucleated cells budding from the periphery (arrows) were seen in a sheet-like coenocyte stained with Neutral Red vital stain. B) Complex networking (arrowheads) between cellular bodies was evident in an unstained arachnoid coenocyte. Scale bar: 30 µm.

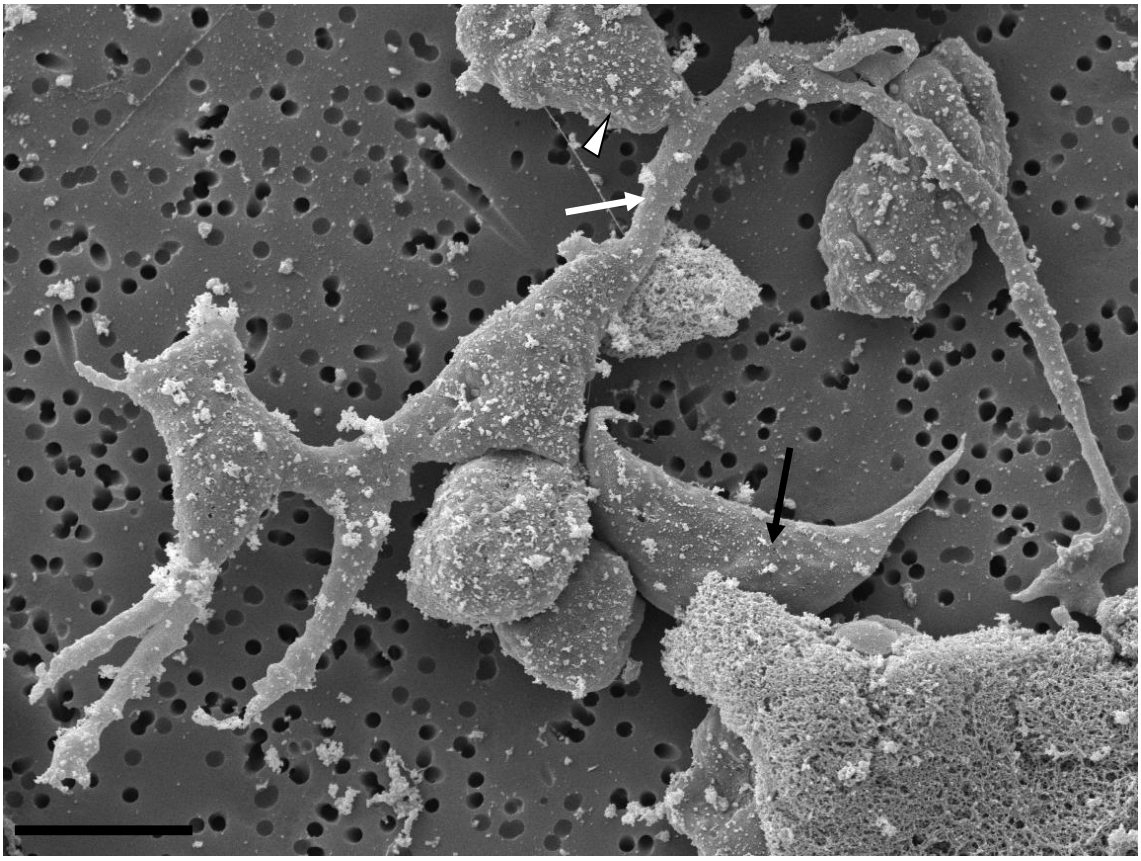

Figure 2.7: Scanning electron micrograph of an arachnoid coenocyte after one week *in vitro.* Finger-like projections (white arrow) extended and connected cellular bodies (white arrowhead). A host hemocyte was also seen adjacent to the coenocyte (black arrow). Scale bar: 10 µm.

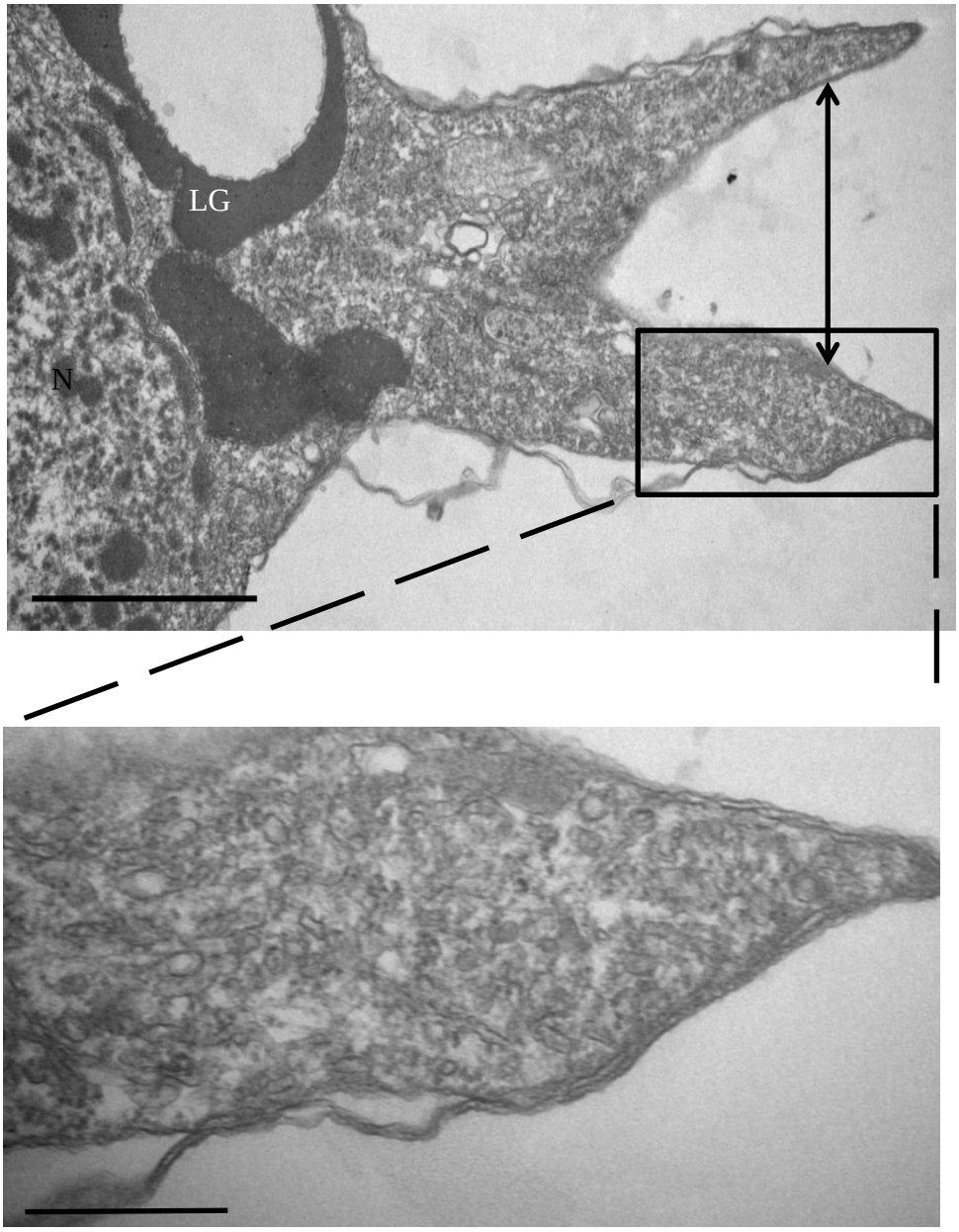

Figure 2.8: Transmission electron micrographs of finger-like projections from an arachnoid coenocyte one week *in vitro.* In the top image, two finger-like projections (double sided arrow) arose from the body of the cell. The projections differed ultrastructurally from the cellular body as they lacked lipofuscin-like granules (LG) and lipid droplets. Lack of microfilaments or microtubules in the projections was evident from a high magnification image (inset). Scale bar: top = 2 μm; inset = 500 nm.

stages (Figure 2.9). Unless viewed ultrastructurally, there was no basis for differentiation. As time elapsed, the outer amphiesmal vesicle membrane and the plasma membrane tightened around the cell, forming spherical cells (Figure 2.10). Concurrent to the tightening of membranes was the rapid proliferation of cells. One sporont gave rise to four daughter cells (Figure 2.11) similar to that described by Appleton and Vickerman (1998). Each daughter cell contained trichocysts and was morphologically and ultrastructurally similar to other sporonts. Cultures seeded with sporonts were the only ones in which further progression to dinospore stages was observed.

2.3.2.4 Dinospores

Ellipsoid dinospores measuring ~14 µm long and 11 µm wide (range 12-16 µm long and 9.5-13 µm wide; n=50) were observed (Figure 2.12). All dinospores were uninucleate, often with nuclear membranes separating, revealing small filaments (Figure 2.13). Dinospores had two flagella, one longitudinal and one transverse, which both arose from the base of the cell, having typical 9 + 2 arrangement of microtubules. Cells were characterized as dinospores by observation of flagella ultrastructurally or motility in culture. Dinospores arose relatively synchronously after ~ one month in culture, with most cells transforming from sporonts to dinospores one week after the first dinospore was observed. Furthermore, motility was observed in cultures incubated at 4°C one week earlier than those incubated at 0°C. Surprisingly, dinospores were observed by TEM within dense aggregates of cells that were fixed one week prior to dinospore motility in culture (Figure 2.14).

2.3.2.5 Post-Dinospore

Hematodinium transitioned to a post-dinospore stage after ~2 weeks as

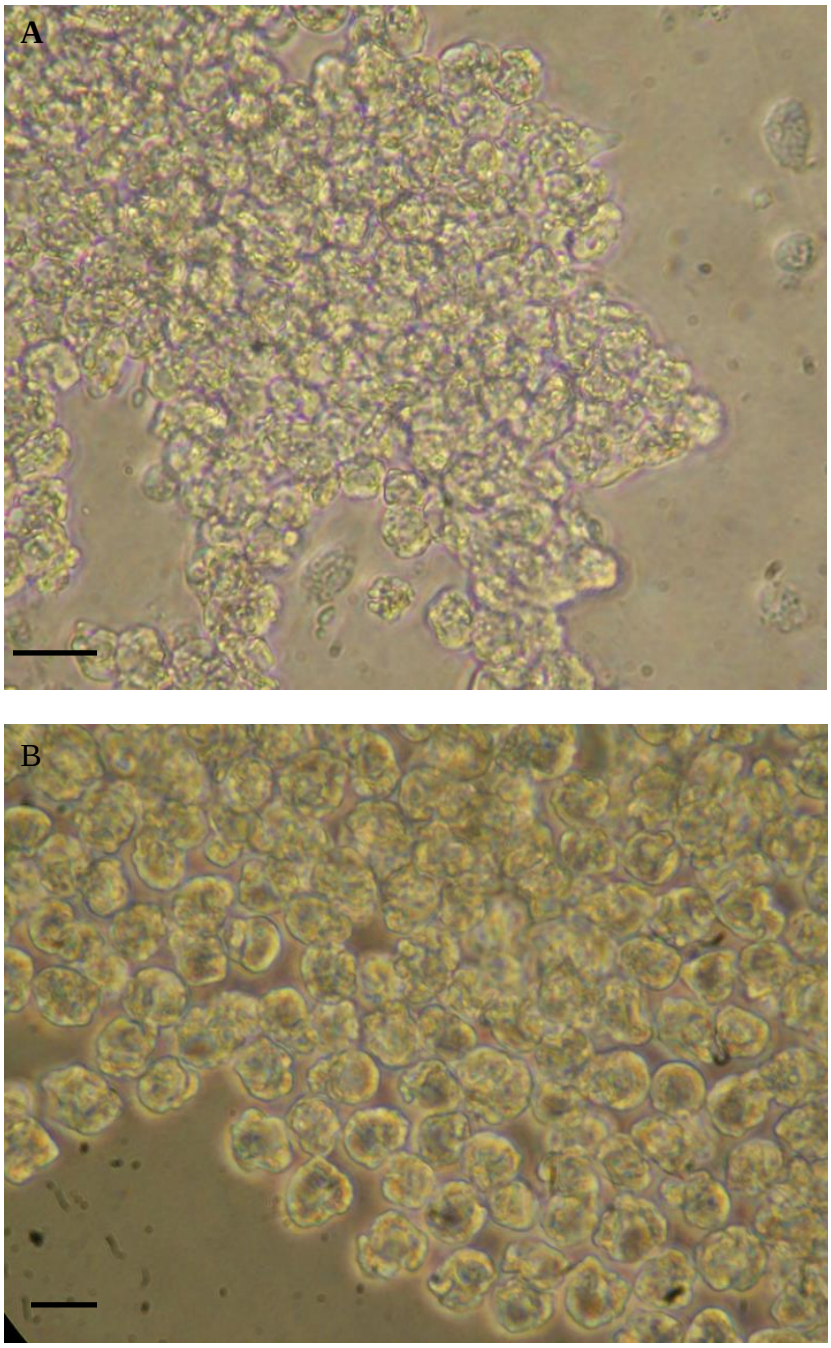

Figure 2.9: Aggregated clusters of *Hematodinium* from *in vitro* culture. A) Aggregated amoeboid trophonts with their irregular morphology were seen by light microscopy. Scale bar: 30 µm. B) Aggregated sporonts showed similar morphology to amoeboid trophonts, and were indistinguishable from amoeboid trophonts when viewed by light microscopy. Scale bar: 15 µm.

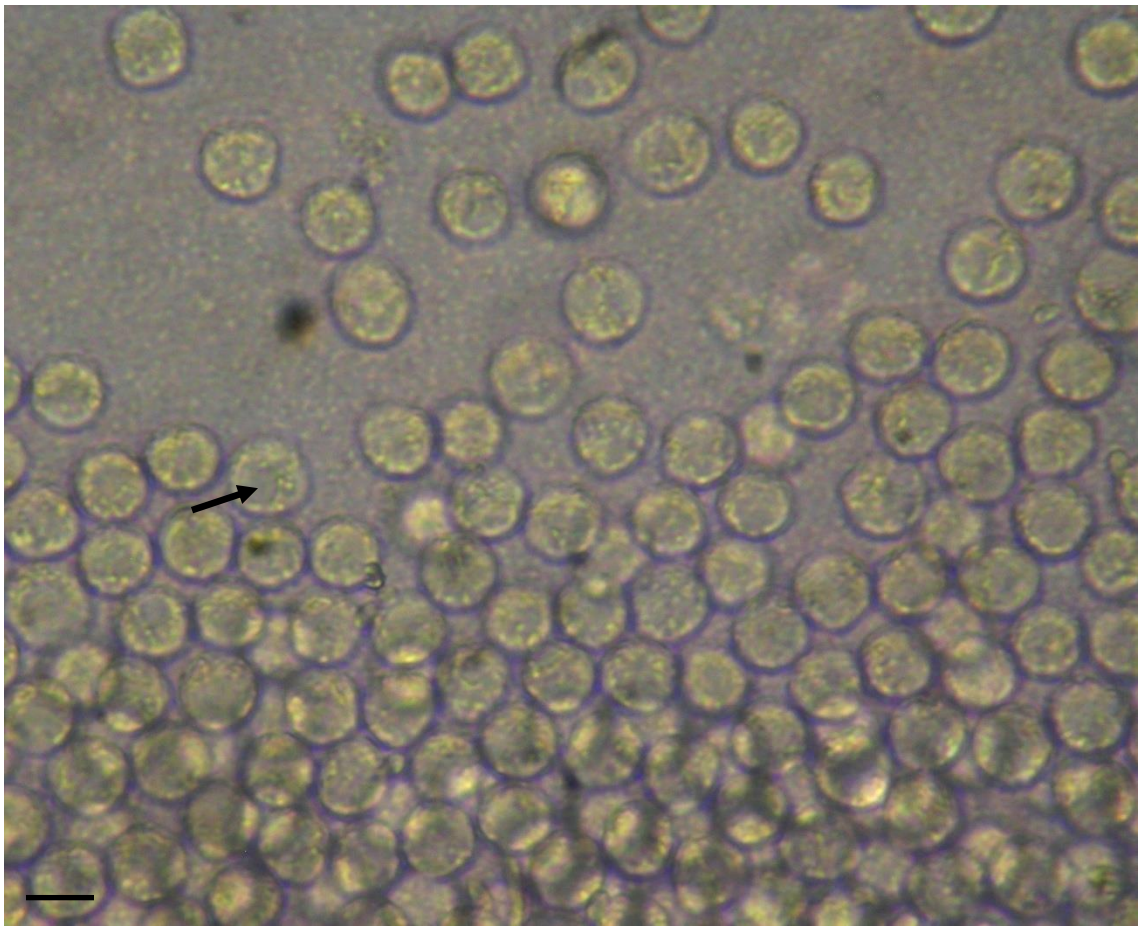

Figure 2.10: Dense aggregates of late stage sporonts had spherical morphology when viewed under light microscopy after three weeks *in vitro.* Notably, these sporonts were exclusively uninucleated (arrow). Scale bar: 10 µm.

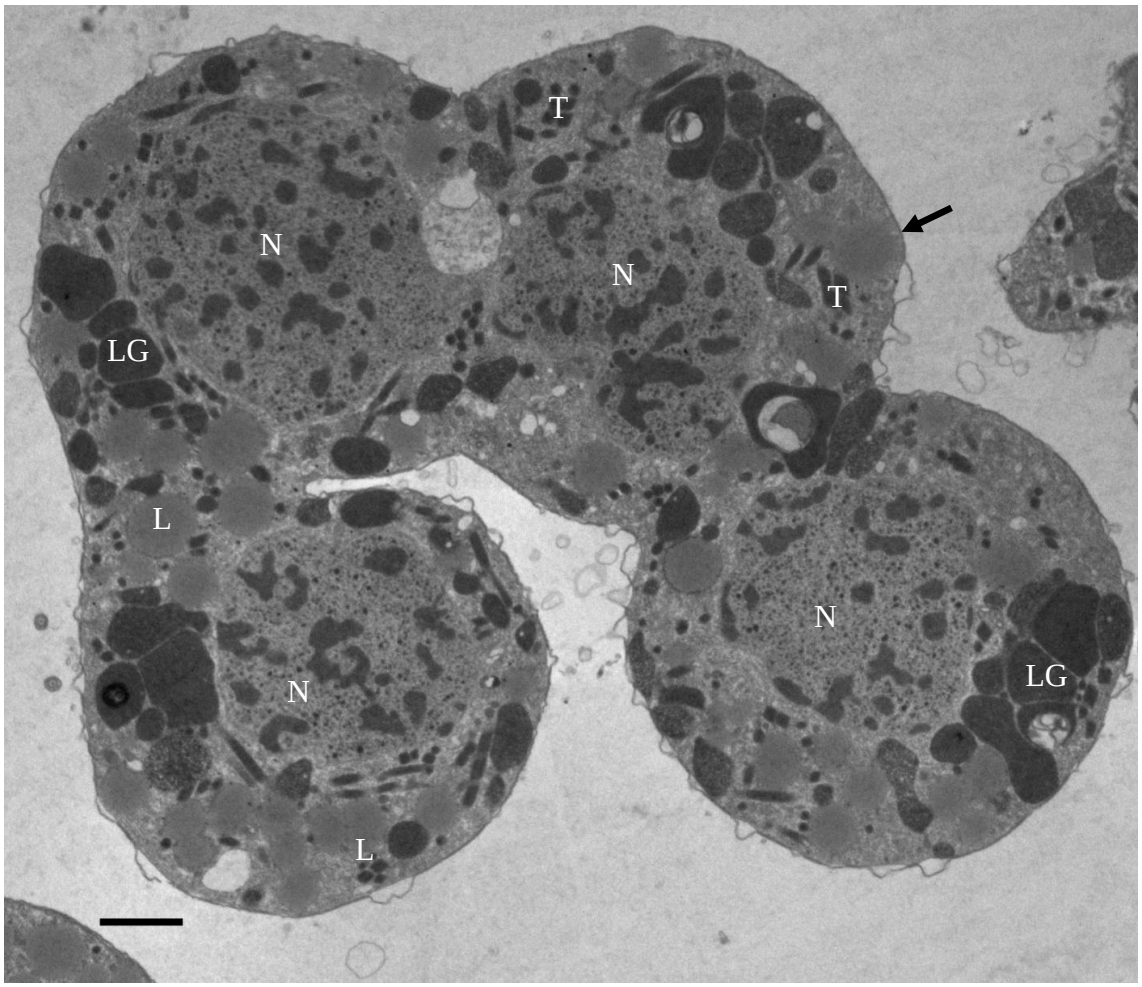

Figure 2.11: A transmission electron micrograph of a late stage sporont dividing into four uninucleated daughter cells. Note the close association between the amphiesmal membranes and the plasma membrane (arrow), resulting in a spherical morphology of cells. T = trichocysts, N = nucleus, L = lipid droplet, LG = Lipofuscin-like granules. Scale bar: 2 µm.

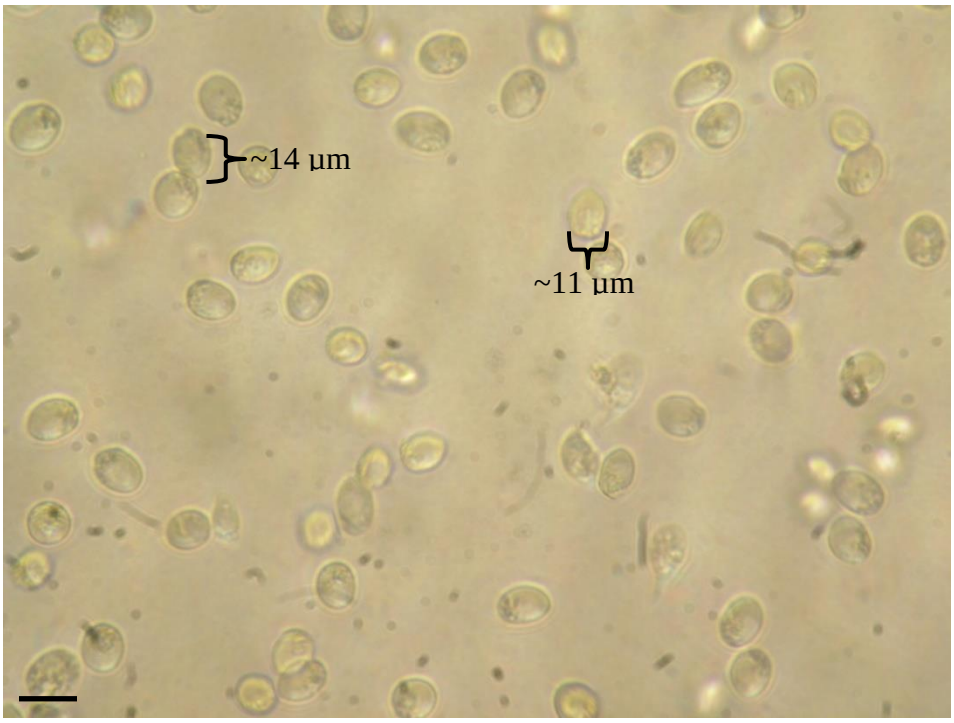

Figure 2.12: Uninucleate and ellipsoid macrodinospores measuring ~14 µm long and ~11µm wide and ranging between 12-16 µm long and 9.5-13 µm wide respectively were seen by light microscopy after ~ one month *in vitro*. Scale bar: 15 µm.

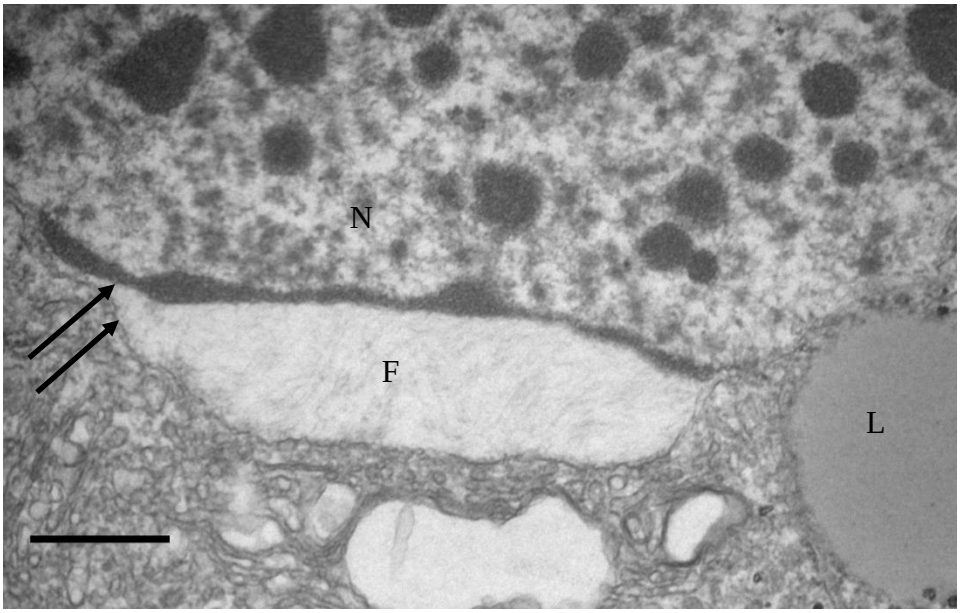

Figure 2.13: Transmission electron micrograph of a mature dinospore. Separation of nuclear membranes (arrows) was evident and filamentous material (F) was observed within the intermembranous space. N = nucleus, L=lipid droplet. Scale bar: 500 nm.

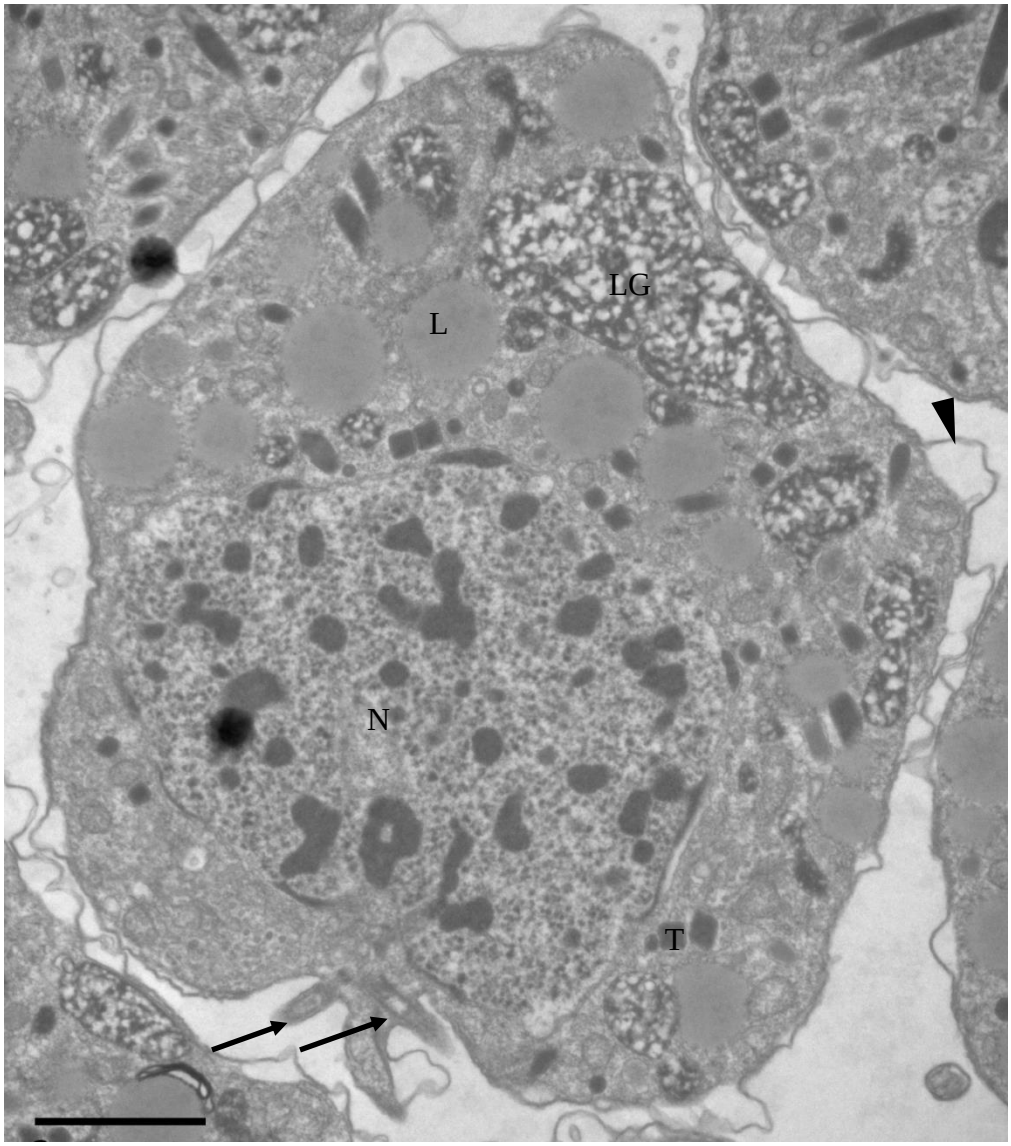

Figure 2.14: A mature dinospore was seen within a dense cluster of cells one week prior to motility *in vitro.* Two flagella (arrows) were evident at one end of the cell, the nucleus (N) was positioned centrally, and partially degranulated lipofuscin-like granules (LG) were located at the other end of the cell. Trichocysts (T) and lipid droplets (L) were found ubiquitously throughout the cell, and the plasma membrane (arrowhead) was seen bulging from the cell. Scale bar: 2 µm.

dinospores. Post-dinospores lost their flagella becoming non-motile, and became spherical, similar to late sporont stages (Figure 2.15). Nuclear ultrastructure changed with beaded chromatin observed primarily along the periphery of the nucleus, with a single bead of heterochromatin in the centre of the nucleus (Figure 2.16). Trichocysts remained although some were eventually observed in autophagosomes (presented and discussed in Chapter 3). Based on these characteristics, they were not classified as trophonts or sporonts, but as post-dinospores. Although the cells were viable they did not develop to trophonts or sporonts.

2.3.2.6 Schizont

In numerous cultures, schizonts developed. Schizonts were morphologically and ultrastructurally similar to schizonts described in Year 1, and were characterized as large cells containing a large vacuole with peripheral clumps of lipofuscin-like material. As in 2010, these forms arose concurrent to contamination, but were also observed in non-contaminated cultures that had been sustained for long periods (> 3 months).

2.3.2.7 Senescent clump colonies

Shortly after initiation of cultures #62 and #63, clumps arose in both cultures. Initially these were assumed to be dense aggregates of cells or large coenocytes, as individual amoeboid trophont cells surrounded clumps when observed with light microscopy (Figure 2.17); however, these clumps were deemed senescent via ultrastructural analysis. Membrane bound cells were not evident, and nuclear structure was absent within clumps. Clumps consisted of membranous material, lipid droplets, and other degraded organelles (Figure 2.18) resembling cellular debris from culture.

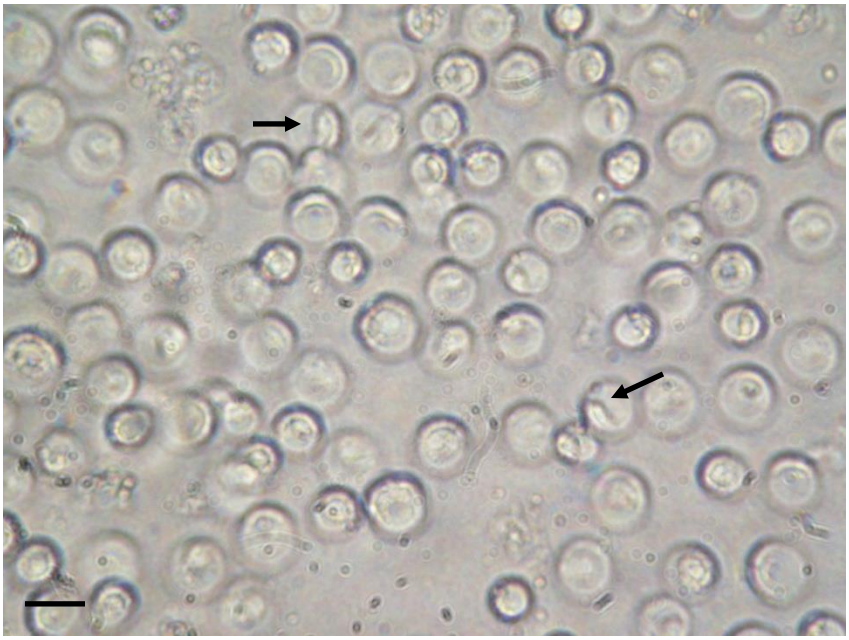

Figure 2.15: Post-dinospores observed by light microscopy ~2 weeks after dinospore formation *in vitro*. *Hematodinium* lost their flagella, became non-motile and rested on the bottom of the culture well. Notably, cells were spherical and uninucleated, with the nucleus (arrow) occupying most of the volume of the cell. Scale bar: 10 µm.

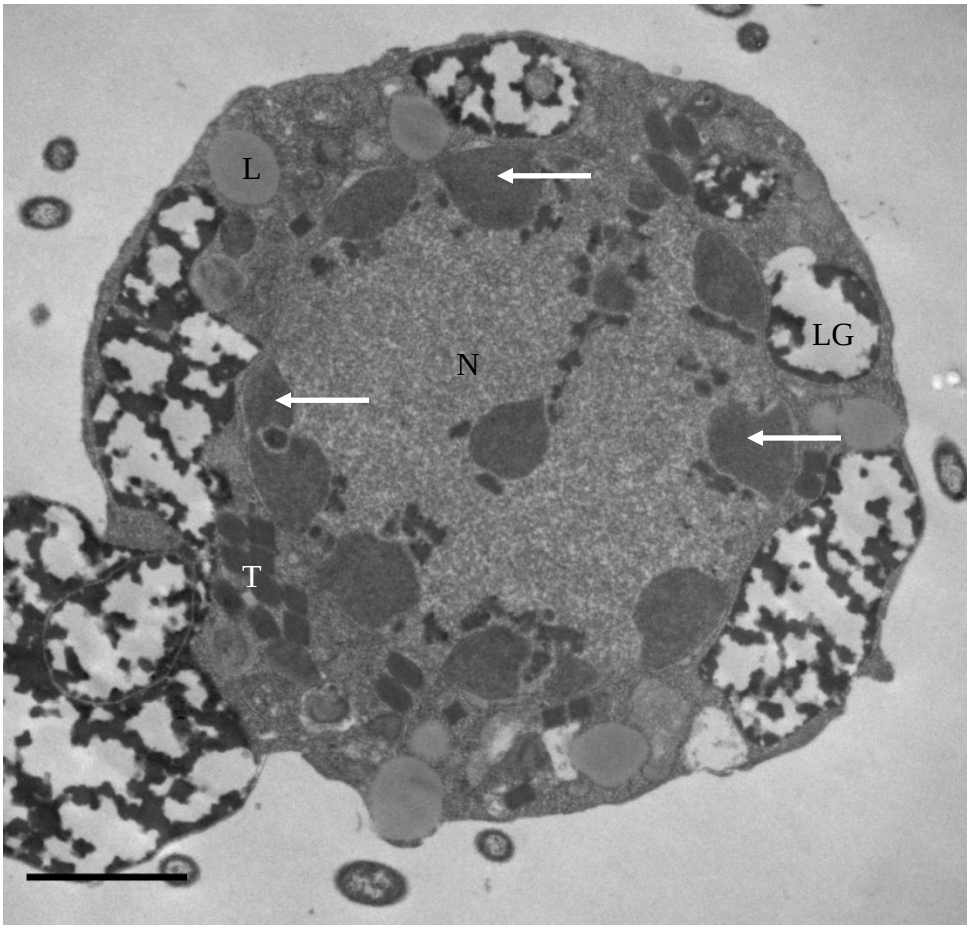

Figure 2.16: Transmission electron micrograph of post-dinospore stage. A single bead of heterochromatin was found in the central region of the nucleus (N), with all other heterochromatin lining the periphery (arrows). The nucleus occupied much of the volume within the cell, but lipid droplets (L), trichocysts (T), and degranulated lipofuscin-like granules (LG) were also evident. Scale bar: 2 µm.

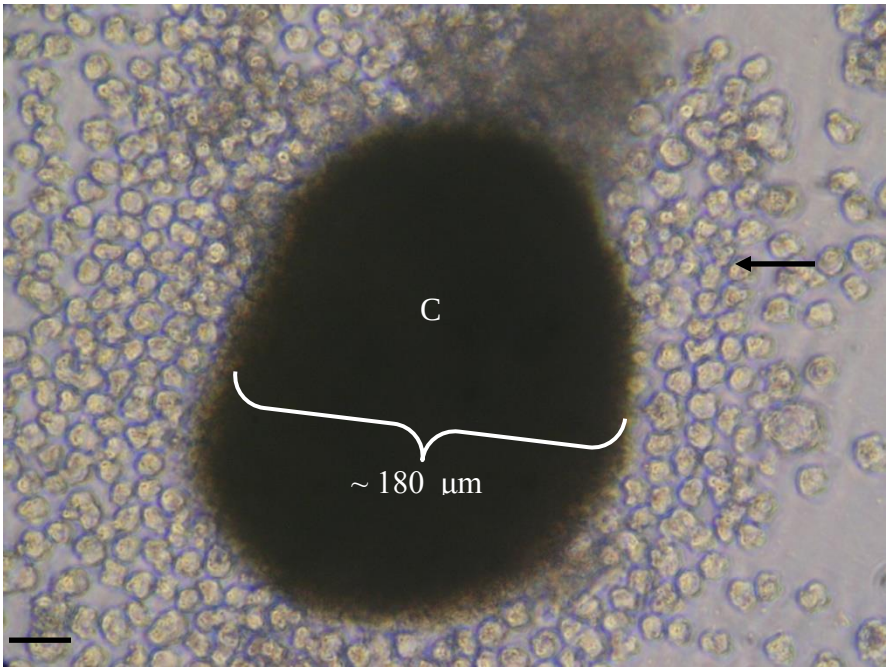

Figure 2.17: A large, dense clump (C) measuring ~180 µm wide was seen *in vitro* by light microscopy. Additionally, aggregates of trophonts (arrow) were adjacent to the large clump. Scale bar: 30 µm.

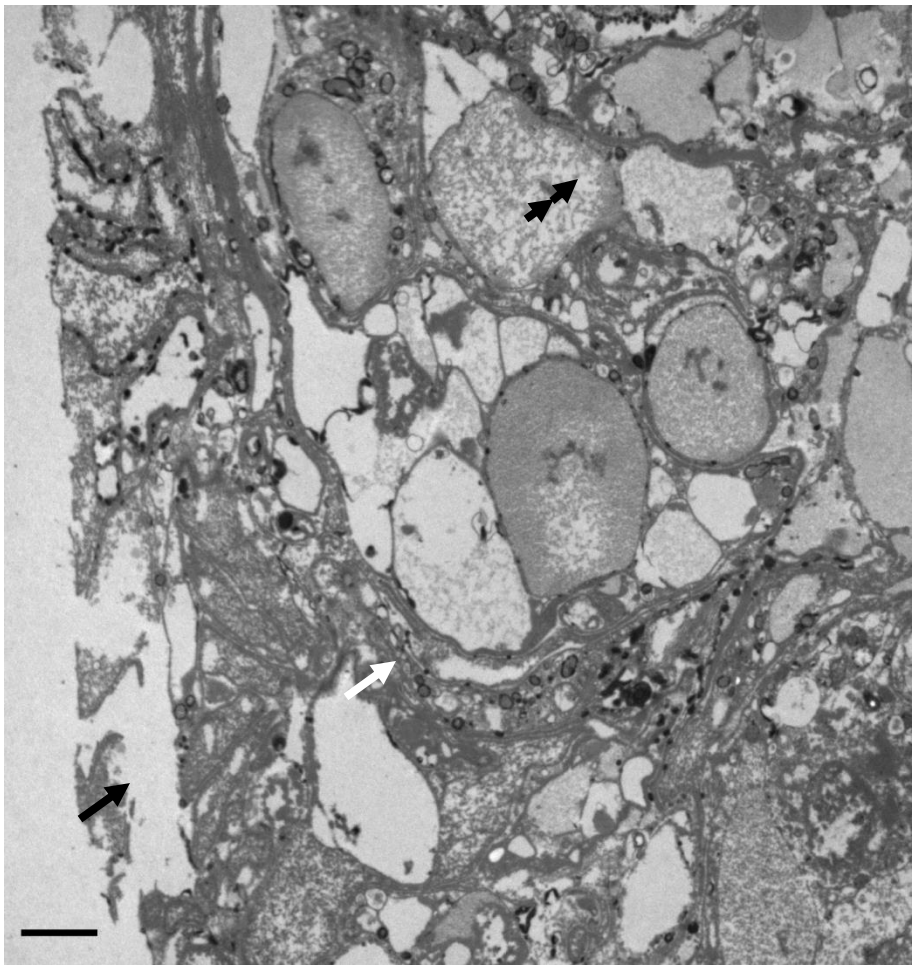

Figure 2.18: Transmission electron micrograph of a clump revealed lack of nuclear and cellular integrity in this structure. The clump consisted of aggregated membranous material (white arrow), and remnants of organelles (double arrow). Furthermore, there were no clear boundaries between the clump and its surrounding environment (black arrow). Scale bar: 2 µm.

2.3.2.8 Lipofuscin-like granules

In all cultures and in all life stages, lipofuscin-like granules were seen in *Hematodinium*. Lipofuscin is composed of oxidized proteins and lipids found within lysosomes which apparently cannot be digested (Terman and Brunk, 2004). Ultrastructurally, granules containing heterogeneous granular material were surrounded by a single membrane (Figure 2.19). Cytoplasm specific autofluorescence was observed in direct smears excited with ultraviolet light (Figure 2.20). Many areas of the cytoplasm stained orange and red with Acridine Orange and Neutral Red respectively (Figure 2.21), indicative of an acidic organelle such as the lysosome. Shortly after cultures were seeded, lipofuscin-like granules appeared to degranulate and this continued throughout *in vitro* cultivation. Lipofuscin-like granules were 14.35 ± 0.40 nm (n=100) in diameter in initial hemolymph samples, and were significantly larger after one month $p<0.001$, measuring 24.10 ± 0.73 nm (n=100), often having a 'halo' appearance. Varying degrees of degranulation were apparent in individual cells (Figure 2.22). The mechanism of degranulation is unclear. When stained with Acridine Orange, red globules were seen extracellularly (Figure 2.21). As time in cultures progressed, the number of lipofuscin-like granules decreased; however the size of vesicles increased. At the end of the 16 week trial, most *Hematodinium* contained only one large, relatively empty, vesicle containing peripheral clumps of granular lipofuscin-like material characteristic of schizonts.

2.3.2.9 Filopodia-like structures

Filopodia-like structures were observed in several early sporonts and amoeboid trophonts (Figure 2.23). Filopodia extended considerable distances away from the

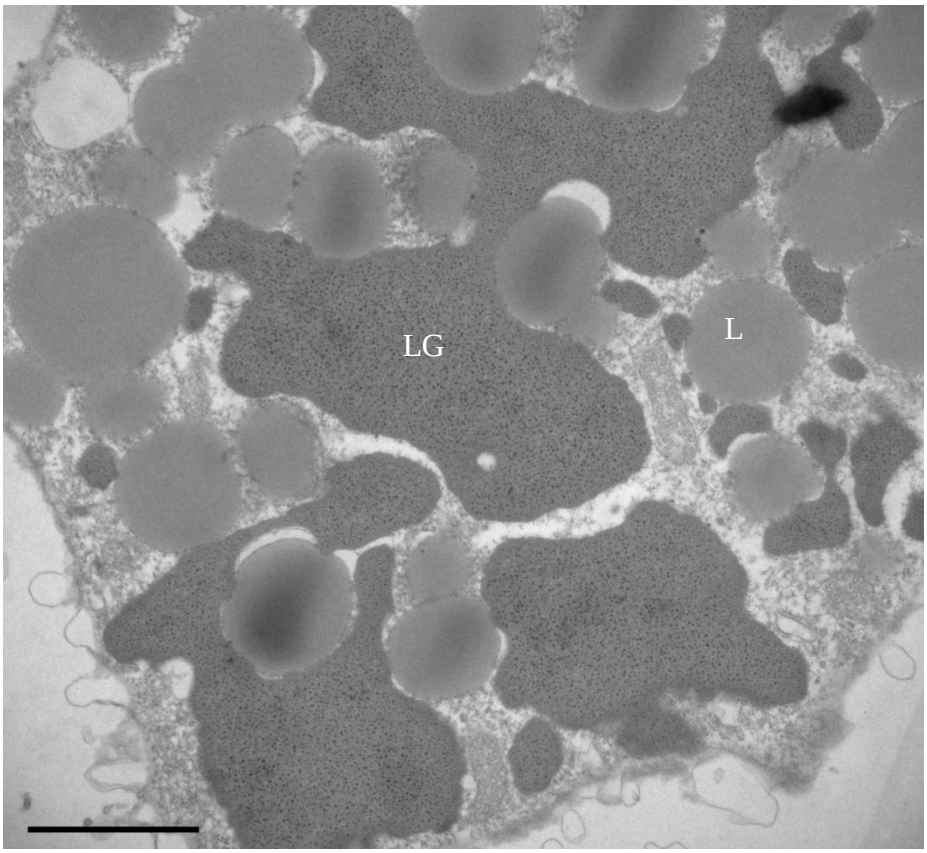

Figure 2.19: Transmission electron micrograph of an amoeboid trophont ~3 weeks *in vitro*. Heterogeneous, electron dense lipofuscin-like granules (LG) were contained in large, single membrane vesicles that were usually non-spherical and varied in shape. These were often seen adjacent to lipid droplets (L). Scale bar: 2 µm.

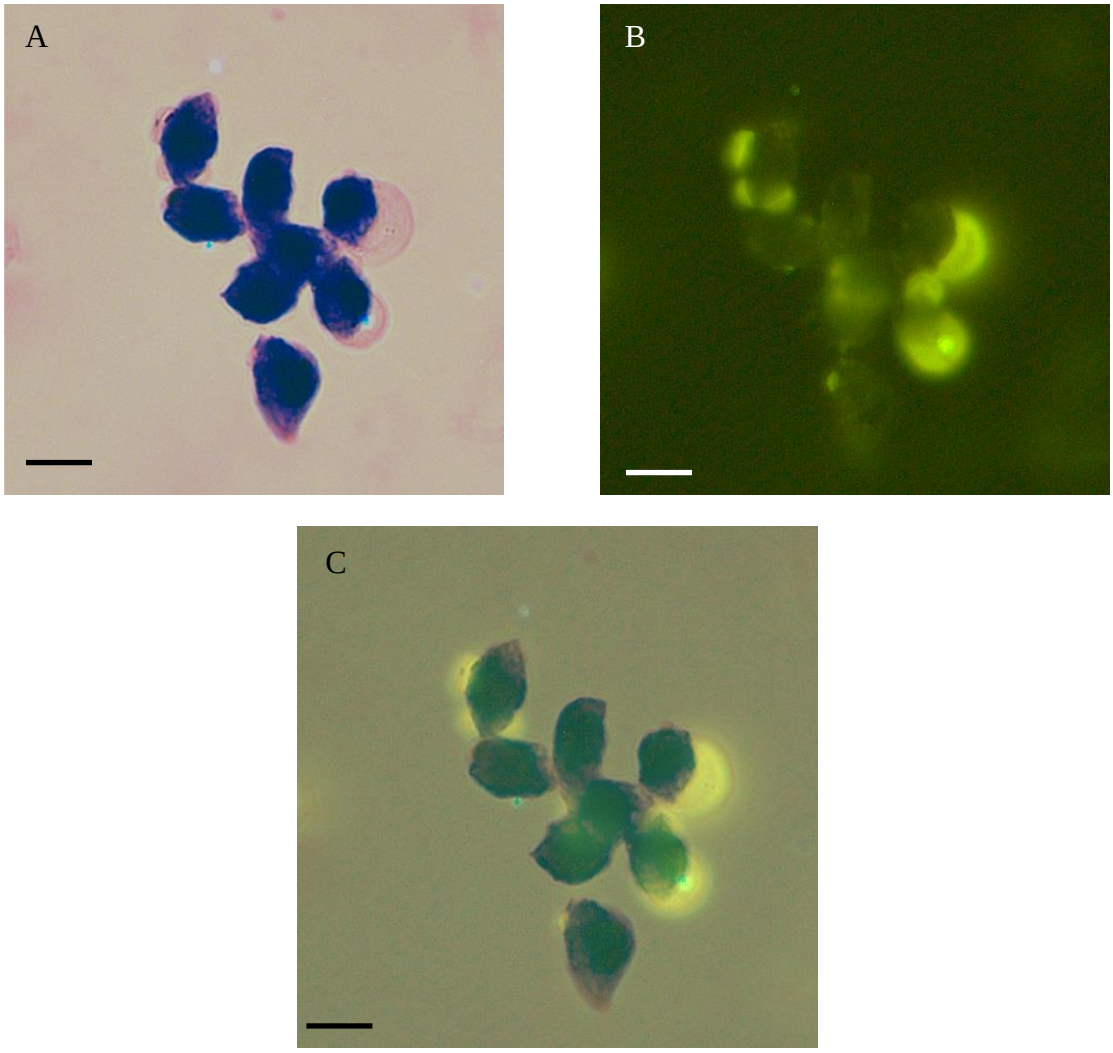

Figure 2.20: Direct smear of *Hematodinium* sporonts after two weeks *in vitro.* A) *Hematodinium* stained with Wright-Giemsa reveal dense chromatin (purple) within nuclei. B) When viewed with ultraviolet light passed through an FITC filter, autofluorescent pigments (green) were clearly seen. C) When images 'A' and 'B' were merged, it was evident that the autofluorescent pigments were restricted to the cytoplasm of cells. Scale bar: 10 µm.

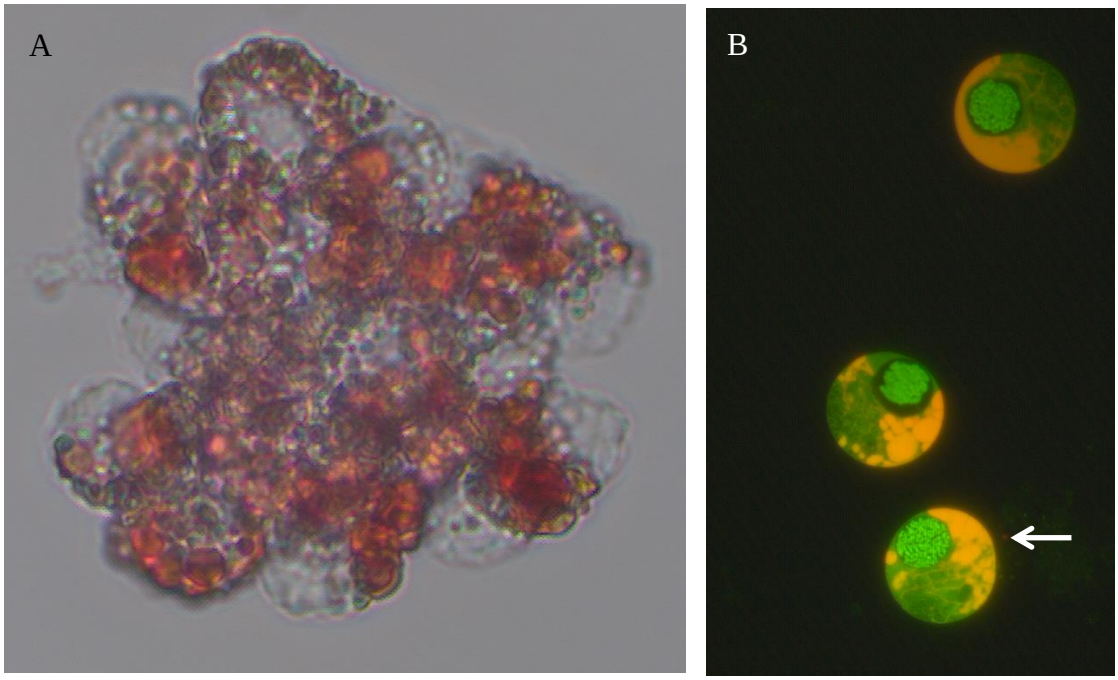

Figure 2.21: Amoeboid trophonts and sporonts vitally stained after one month *in vitro* and viewed by light microscopy. A) An aggregate of amoeboid trophonts was stained with Neutral Red, revealing many lysosomes (red) throughout the cells. B) Numerous cytoplasmic contents of sporonts vitally stained with Acridine Orange fluoresce orange, indicative of acidic organelles such as lysosomes. Note a small orange globule present extracellularly (arrow). Permanently condensed chromatin, characteristic of dinoflagellates, were stained green within the nucleus. Scale bar: 10 µm.

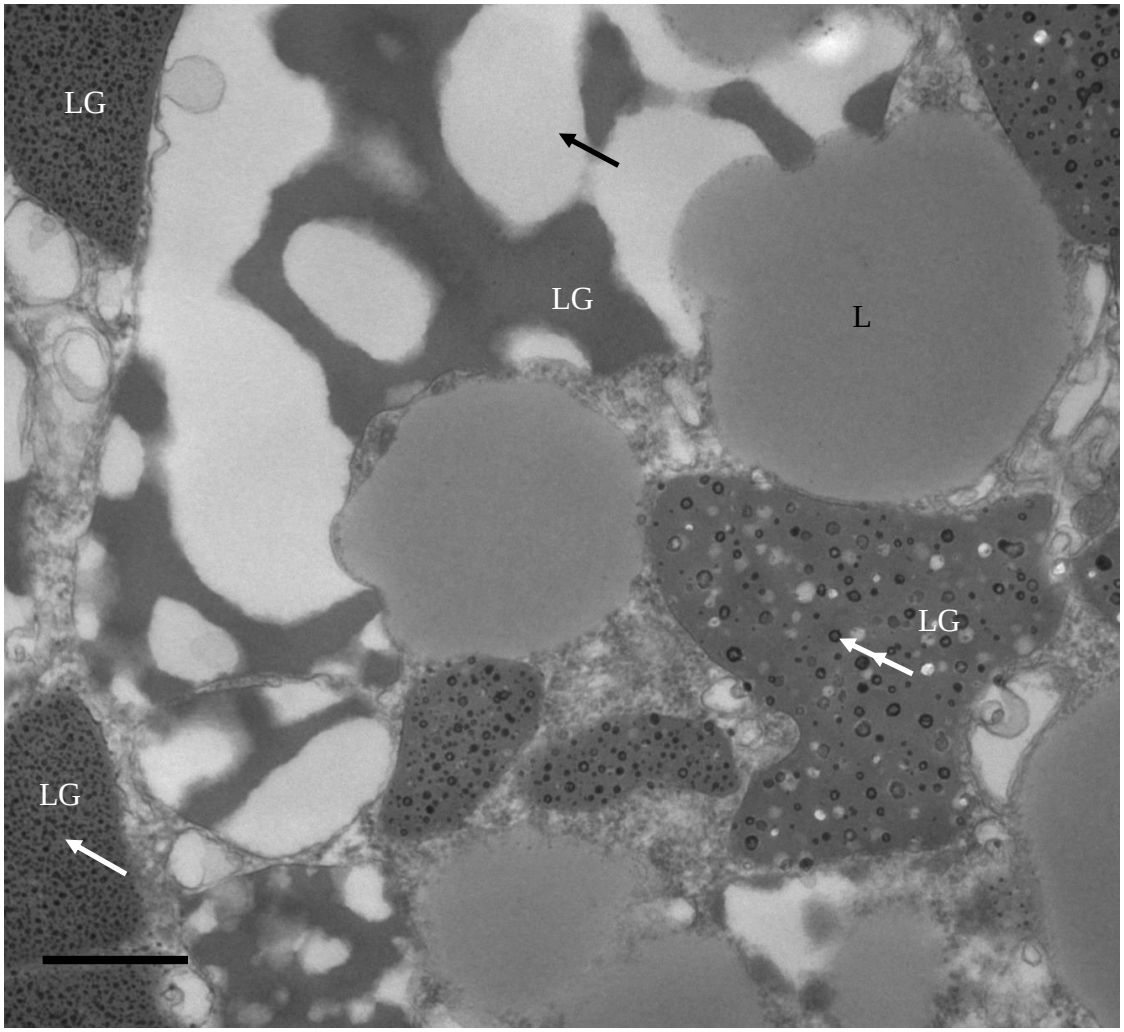

Figure 2.22: Amoeboid trophont, viewed by transmission electron microscopy, showed varying degrees of lipofuscin-like granule (LG) degradation. Some lipofuscin-like material showed little degranulation (white arrow) while others showed moderate (double white arrow), and much (black arrow) degranulation. Regions of granules became less electron dense as degranulation occurs, leaving a 'halo' appearance to some material (double white arrow). As degranulation progressed, large electron lucent areas within the vesicle were seen (black arrow). L = Lipid body. Scale bar: 500 nm.

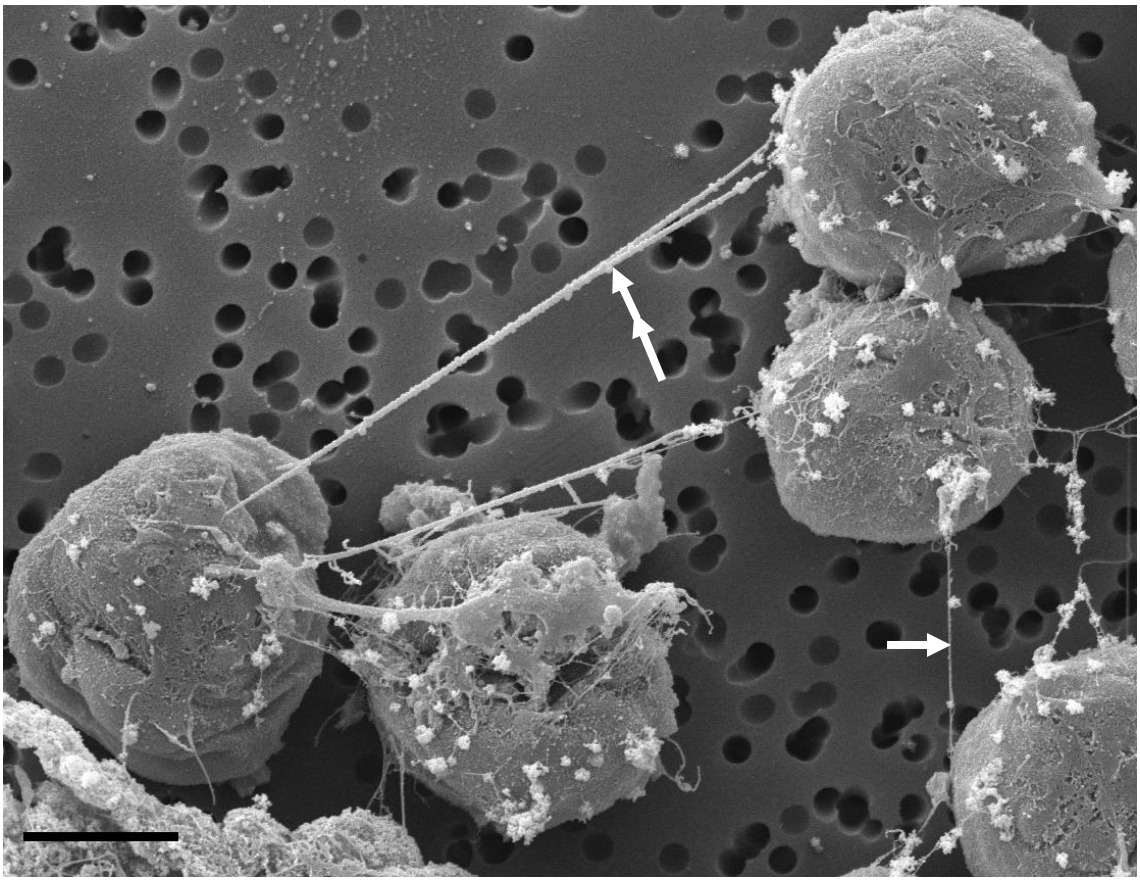

Figure 2.23: A scanning electron micrograph of sporonts showed filopodia-like structures attaching to neighbouring cells (arrow). Filopodia-like structures attaching to the filter membrane (double arrow) were likely attached to a neighbouring cell but collapsed during desiccation in SEM processing. Scale bar: 5µm.

organism and often attached to neighbouring cells. When vital stained, filopodia seemed to flutter in the medium; however, when kept in direct fluorescent light the fluttering stopped and the apparatus contracted (Figure 2.24).

2.4 Discussion

2.4.1 Culture media

In 2010, there was no growth in modified ciliate medium or DMEM: Hams F12 media, although there was sustained viability in modified ciliate medium. *Nephrops* medium yielded better results; sustained viability and life stage progression to schizont forms was observed. Only *Nephrops* medium was used in 2011. *Nephrops* medium has been used successfully in the two previous *in vitro* studies of *Hematodinium* (Appleton and Vickerman, 1998; Li *et al*., 2011). Li *et al*. (2011) modified the existing *Nephrops* medium with supplementation of blue crab serum; however, *Hematodinium* growth and viability did not improve despite supplementation (Jeffrey Shields, personal communication). Consequently, due to the costly nature of rearing Atlantic snow crabs and possibility of introducing contaminating organisms, supplementation of the medium with crab serum was not performed.

The cause of *Nephrops* medium's superiority over other media is unknown. The other media may have contained components inhibitory to *Hematodinium* growth and development. Another possibility is that specific electrolyte balances may play a significant role in parasite survivability. Perhaps *Nephrops* medium more closely mimicked the environment in crustaceans, making it a superior medium.

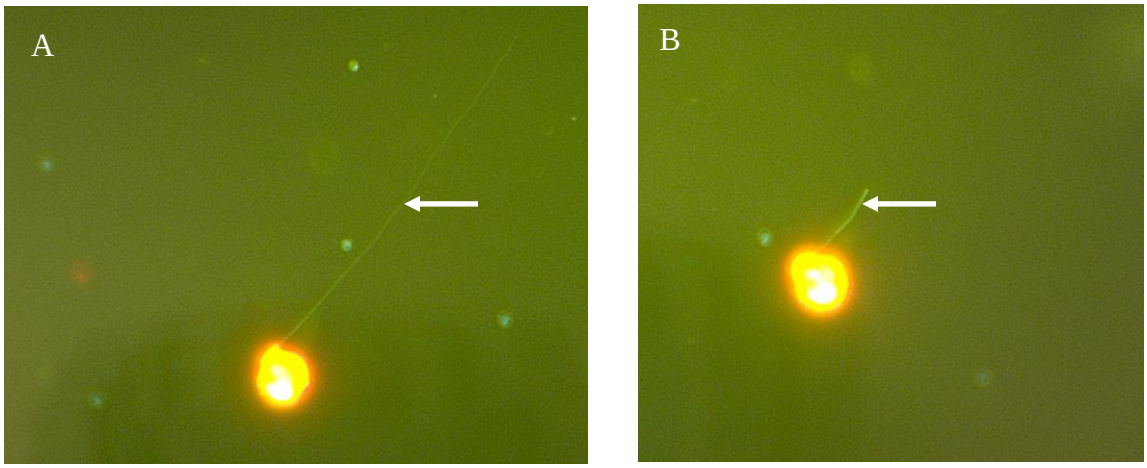

Figure 2.24: Amoeboid trophonts, stained with Acridine Orange, two weeks *in vitro*. A) A filopodia-like structure (arrow), several times the length of the cell, was seen extending into the environment. B) After being exposed to ultraviolet light for several seconds, the filopodia-like structure (arrow) contracted and was notably shorter. Scale bar: 10 µm.

2.4.2 Hematodinium life stages

A total of six *Hematodinium* life stages were observed during *in vitro* culture, the most common being the amoeboid trophont stage. This stage appeared in all but two crab hemolymph samples from 2011 and often did not progress to other developmental stages for the duration of culturing. Amoeboid trophonts were replicative, as cultures often became dense and required dilution throughout the trial. They were occasionally uninucleated, often multinucleated, and possessed highly variable morphology. Trophonts were seen individually, as part of large aggregations of individual cells, or adjacent to coenocytes. There were two morphological types of trophonts. Some appeared filamentous, similar to those described previously (Appleton and Vickerman, 1998; Li *et al*., 2011), whereas others were irregular in shape, as imperfect ellipsoids or spheres. The irregular morphologies of amoeboid trophonts (seen in light and scanning electron micrographs) are likely the result of the irregular bulging of the plasma membrane.

In both morphological types of trophonts, there were no ultrastructural differences. Both had similar nuclear patterns, numerous lipid droplets, and lipofuscin-like granules. Also, both lacked trichocysts. Stages that resemble filamentous forms are considered morphological variations of amoeboid trophonts. Writhing filamentous forms as described in blue crabs and Norway lobsters (Appleton and Vickerman, 1998; Li *et al*., 2011) were not observed.

Amoeboid trophonts are likely the result of budding from sheet-like coenocytes. After these amoeboid stages bud from the central coenocyte mass, they likely undergo nuclear division without accompanying cytokinesis, resulting in a newly formed sheet-

like coenocyte, from which multiple amoeboid trophonts may bud. This pattern may occur multiple times, resulting in exponential growth of *Hematodinium*. Appleton and Vickerman (1998) and Li *et al*., (2011) describe a similar developmental stage and refer to them as 'clump colonies'. However, the multinucleated masses observed were sheet-like, and never clump-like. This fits well with *in vivo* descriptions of sheet-like stages from histopathological studies of *Hematodinium*-infected crustaceans (Field and Appleton, 1995; Wheeler *et al*., 2007; Chualáin and Robinson, 2011). These multinucleate masses have been termed coenocytes rather than syncytium or plasmodium, as described previously (Appleton and Vickerman, 1998; Stentiford and Shields, 2005; Li *et al*., 2011) since they are likely the result of nuclear division with unaccompanied cytokinesis rather than fusion of cells (Baluška, 2004). Trophont coenocyte attachment to the substratum was not observed, unlike the arachnoid trophont stage described by Appleton and Vickerman (1998) and Li *et al*., (2011). Perhaps attachment was not observed due to the dense aggregation of cells surrounding coenocytes in culture.

Dense colonies were observed by light microscopy that resembled 'clump colonies' described by Appleton and Vickerman (1998) and Li *et al*., (2011). However, when viewed ultrastructurally, this mass was senescent. There were no clear cellular membranes or nuclear integrity. These clumps were likely coalesced aggregates of cellular debris.

2.4.3 Sporonts

All hemolymph samples from 2010 and two hemolymph samples from 2011 contained *Hematodinium* sporonts. Sporonts often resembled amoeboid trophonts in their

light microscopic morphology (Figure 2.9), but contained trichocysts when viewed ultrastructurally. Trichocysts are rigid extrusion organelles, discussed in Chapter 3. Sporonts, like amoeboid trophonts, were seen individually, as part of aggregates, or adjacent to coenocytes. Coenocytes adjacent to sporonts differed from the coenocytes described above as they contained trichocysts and sometimes had distinct appendages that attached to the culture well (Figure 2.6, B). This supports similar distinctions between trophont and sporont forms of multinucleated plasmodia (Appleton and Vickerman, 1998; Li *et al*., 2011). Some coenocytes were sheet-like, whereas others resembled arachnoid sporont stages described by Appleton and Vickerman (1998) and Li *et al*., (2011), thus they are termed arachnoid coenocytes.

Sporonts were observed adjacent to these coenocytes; thus, sporonts likely bud from coenocytes through fission, similar to the amoeboid trophonts above. The interconnected mass was most evident along the periphery of the coenocytes, where cells were less dense (Figure 2.6, B). Sporonts and arachnoid coenocytes observed in collected hemolymph and early cultures were likely early forms of their respective stage, since immature and developing trichocysts were seen ultrastructurally (See Chapter 3).

The distinction between early sporont and amoeboid trophont developmental stages was discerned ultrastructurally. Thus, the distinction between arachnoid trophonts and arachnoid sporonts reported by Li *et al*. (2011) is difficult when solely using light microscopy. Furthermore, arachnoid stages were only observed in sporont coenocytes in the present study, contrary to the observations of *Hematodinium* from blue crabs (Li *et al*., 2011). It is also difficult to assess whether aggregates of cells are amoeboid

trophonts or early sporonts by light microscopy; aggregates were observed in both developmental stages.

As sporonts developed, they became uniformly spherical in their morphology, uninucleated, and the outer amphiesmal vesicle and plasma membranes tightened around the cell. Sporont development was progressive in nature with early stages, resembling trophonts morphologically, gradually becoming spherical and uninucleate. Late stage sporonts also replicated to incredible densities within culture, often covering over 2/3 of the well bottom with several 1:1 dilutions needed to achieve reasonable densities for maintaining viability. Sporonts were observed dividing into four daughter cells with transmission electron microscopy. This is similar to descriptions from Appleton and Vickerman (1998).

2.4.4 Dinospores

Approximately one week after late sporont stages were observed, and one month after cultures were initiated, dinospores were observed. As both 2011 sporont cultures eventually gave rise to dinospores, this suggests that *Hematodinium* is committed to proceed to dinospore production after it reaches the sporont developmental stage. Dinospores, similar to sporont stages, contained lipofuscin-like granules, lipid droplets, beaded chromatin, and trichocysts. These cells contained two flagella: one transverse, responsible for the propulsion of the organism, and one longitudinal, responsible for directing the organism (Appleton and Vickerman, 1998). Furthermore, separation of the nuclear envelope was often observed, revealing unknown fibrillar material between membranes (Figure 2.13). Appleton and Vickerman (1998) reported similar findings in sporoblasts, and tentatively identified the fibrillar material as flagellar hairs destined for

the transverse flagellum. It is unlikely that these are flagellar hairs, as fibrillar material was observed in mature dinospores in the present study.

Hematodinium was characterized as a dinospore if flagella were seen ultrastructurally or if motility was observed in culture. Dinospores were ~14 µm long ranging 12-16 µm, and were ~ 11 µm wide ranging 9.5-13 µm (n=50). Two types of *Hematodinium* dinospores, termed macrodinospore and microdinospore have been reported (Appleton and Vickerman, 1998; Stentiford and Shields, 2005; Li *et al*., 2011). Macrodinospores were 14.3 ± 1.6 µm long and microdinospores were 9.2 ± 2.4 µm long (n=25) from *Hematodinium* isolated from *C. sapidus* (Li *et al*., 2011). Dinospores isolated from *N. norvegicus* showed slightly larger dimensions with macrodinospores and microdinospores measuring 16-20 µm and 11-14 µm long (n=25) respectively. Macrodinospores have a characteristic ellipsoid shape, and microdinospores have a 'corkscrew' appearance (Appleton and Vickerman, 1998; Li *et al*., 2011). Thus, the dinospores observed herein were morphologically and dimensionally similar to macrodinospores reported by Li *et al*., (2011). Only one dinospore type was observed, which is consistent with previous work describing a single type arising from an individual crab (Meyers *et al*., 1987; Appleton and Vickerman, 1998; Li *et al*., 2011). The lack of microdinospores arising in cultures is likely the result of the small sample size.

Interestingly, dinospores were found within dense aggregates of cells by TEM one week prior to observing dinospore motility in culture. Due to the cold temperature at which they were incubated (0°C), their metabolic rate was likely low. The density of cultures in which they were found, in combination with a presumably low metabolic rate,

likely made motility difficult; this likely explains why motility was observed one week after dinospores were detected with TEM.

Furthermore, motility was observed one week earlier in cultures incubated at 4°C to those kept at 0°C; samples were not taken from the 4°C cultures prior to observed motility. It is unlikely that progression through developmental stages proceeds much faster at elevated temperatures. Appleton and Vickerman (1998) incubated *Hematodinium* isolated from *N. norvegicus* at 6-10°C and dinospore production took 33 days to occur, on average. Furthermore, Li *et al*. (2011) incubated *Hematodinium* isolated from *C. sapidus* at 23°C and dinospores were only observed 6-9 weeks following culture initiation. In the current study, dinospores were observed approximately one month after culture initiation. As progression through developmental stages was slower at elevated temperature, progression could be time dependent and not temperature dependent. As all three studies used the same culture media, it is likely that the reported variation between dinospore production times is due to the stage at which *Hematodinium* was collected, as the time ranged greatly even within individual studies (Appleton and Vickerman, 1998; Li *et al*., 2011). Thus, in the present study, dinospores may have arisen at the same time, but those in colder temperatures were less metabolically active. Therefore, they were observed later with light microscopy than those in warmer temperatures, but were both observed concurrently with transmission electron microscopy. Life stage development may also be dependent on available nutrients or cell density. Appleton and Vickerman (1998) reported the ability to maintain trophont stages indefinitely if medium was changed regularly (every 2-3 weeks). Furthermore, sporonts are seen in late stages of infections *in vivo*. Late stages of infection are characterized by

numerous parasites within the hemolymph and hemocytopenia and organ malfunction. The host becomes moribund as it does not have the resources to continue basic biological functions. With an inability to perform oxidative respiration, both host and parasites quickly deplete any remaining nutrients available, and it is during this period that sporont and dinospores arise *in vivo*. Thus, time, nutrient availability and cell density may play important roles in the development of *Hematodinium.*

2.4.5 Post-Dinospores

After ~2 weeks, dinospores lost their flagella, becoming non-motile, and their morphology shifted from ellipsoid to spherical. Dinospores transitioned much earlier than those described by Appleton and Vickerman (1998), and since trichocysts remained, did not transition to trophont stages similar to other *in vitro* studies (Appleton and Vickerman 1998; Li *et al*., 2011). Furthermore, the nuclear structure was notably different than any previous stage. In trophont, sporont and dinospore stages, beaded heterochromatin was evident ubiquitous throughout the nucleus. In the post-dinospore stage, beaded heterochromatin was only seen along the periphery of the nucleus additional to one single bead in the centre, and granular euchromatin material was evident throughout the nucleus (Figure 2.16). Although post-dinospores were viable they did not develop to trophonts. Collectively, this likely indicates that these stages were moribund.

Dinospores apparently represent an infective stage of *Hematodinium* (Appleton and Vickerman, 1998; Stentiford and Shields, 2005; Li *et al*., 2011); thus dinospores likely require entry into a new host for transition to the trophont stage. Perhaps *Nephrops* medium lacked the environmental or chemical signals needed to induce successful transition from dinospores to trophont stages for the *Hematodinium* from *C. opilio*.

Appleton and Vickerman (1998) described the need to adjust the cell density to achieve a monolayer of cells to induce further life stage development while Li *et al*. (2011) combined crab hemolymph with medium throughout culturing. The addition of hemolymph in culture may more closely mimic the environmental and/or chemical setting whereby life stage progression occurs within a crustacean host. As transition is possibly time dependent, as noted above, transition likely began irrespective of environmental or chemical cues; however, proper environmental or chemical stimuli may be needed to successfully complete transition.

2.4.6 Schizont

Large schizonts measuring up to 60 µm in diameter were observed in both 2010 and 2011 cultures and were seen with and without trichocysts. They were characterized by their large size and large, relatively empty vacuole containing peripheral clumps of lipofuscin-like material. Schizonts were only observed in older cultures, and often in cultures with bacterial or yeast contamination. This stage likely does not represent a normal life stage of *Hematodinium* but arises only when severely stressed. The schizont is considered an aberrant form of sporonts and trophonts. Schizonts were indicative of the overall health of the culture, because they were only seen during unfavourable environmental conditions.

2.4.7 Filopodia-like structures

Filopodia-like structures were observed in several early sporonts and amoeboid trophonts, extended considerable distance away from the organism, and often attached to neighbouring cells (Figures 2.23 and 2.24). When vitally stained, filopodia seemed to flutter in the medium, but contracted when held in direct fluorescent light. Since these

structures were seen attaching to neighbouring *Hematodinium*, they may serve a role in cell-cell communication. Appleton and Vickerman (1998) describe dinospores only transitioning to trophonts if a monolayer is achieved, and arachnoid stages were induced by diluting the culture. Perhaps these filopodia-like structures are able to recognize neighbouring *Hematodinium* and play a role in the progression of life stages. *Hematodinium* was often seen in aggregates near the centre of the well with few cells along the periphery, even after cultures were diluted and cells spread uniformly. Filopodia-like structures may extend from *Hematodinium* in search of neighbouring cells, and upon discovery attach to the neighbouring cell and contract, pulling both cells together. Thus, filopodia-like structures may serve a role in cell motility. However, aggregations may be the result of a separate and unrelated mechanism such as the swirling of media when handled. Swirling may disrupt cells, producing an aggregate near the centre.

As filopodia-like structures attach to neighbouring cells, they may play a role in parasite transmission or tissue attachment and invasion. Early during infection, *Hematodinium* is often seen associated with the hepatopancreas, and may spread to other organs including the gills, heart, skeletal muscle, gut, and others (Field *et al*., 1992; Stentiford and Shields, 2005). How the parasite attaches or associates with these organs is not well understood. Perhaps filopodia-like structures are the means by which *Hematodinium* attach to organs. As the filopodia-like structure can contract, it may also aid in the spread of *Hematodinium* to other organs as well.

2.4.8 Lipofuscin-like granules

Lipofuscin-like granules were seen in all *Hematodinium* stages and often in great quantities. They were ultrastructurally similar to lipofuscin described in vertebrates and invertebrates (Gordon *et al*., 1965; Boellaard and Schlote, 1986; Sarna *et al*., 2003; Vogt, 2012) and are autofluorescent (Figure 2.20). As they were observed in all cells irrespective of life stage, they likely play an important role in *Hematodinium*. In culture, granules degranulated over time as the contents of granules lose electron density, leaving a 'halo' appearance. Electron density continued to dissipate leaving large electron lucent patches within the granule. These granulated vesicles are similar to electron dense deposits described by Appleton and Vickerman (1998), and other granulated vesicles seen from *Hematodinium* isolated from *Chionoecetes tanneri* (Morado, unpublished). The presence of these lipofuscin-like deposits apparently occurs in all isolates of *Hematodinium.*

Lipofuscin is an intralysosomal polymeric material that cannot be degraded by lysosomal hydrolases (Terman and Brunk, 2004). It consists of oxidized proteins and lipids, and accumulates in post-mitotic cells. In vertebrates, lipofuscin forms when hydrogen peroxide (H_2O_2) reacts with ferrous iron (Fe^{2+}) within lysosomes. Hydrogen peroxide is a common product of many oxidative processes within cells, and is often reduced to water through catalases and/or peroxidases. Some H_2O_2 may diffuse into lysosomes, reacting with Fe^{2+} and form the very reactive hydroxyl radical through the following reaction:

$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} OH- + OH\bullet$

Hydroxyl radicals may then react with lipids in the following manner to produce aldehydes (Terman and Brunk, 2004):

$LH + OH\bullet \rightarrow L\bullet + H_2O$

$L\bullet + O_2 \rightarrow LOO\bullet$

$LOO\bullet + LH \rightarrow LOOH + L\bullet$

$LOOH \rightarrow$ various aldehydes

Aldehydes react with free amino groups on proteins, and ultimately result in protein-protein cross-linking via aldehyde bridges (Halliwell and Gutteridge, 1999; Terman and Brunk, 2004). This forms the plastic-like material lipofuscin. Interestingly, elevated lipofuscin levels have been reported in mussels that grew near copper mines (Zorita *et al*., 2006). H_2O_2 likely reacts with Cu^{2+} in a similar manner as above to induce lipofuscin formation.

The oxygen transporting molecule in crustaceans is hemocyanin. Hemocyanin, unlike haemoglobin, is copper based. Furthermore, hemocyanin levels dramatically decrease during *Hematodinium* infection (Love *et al*., 1996) leading to decreased oxygen carrying capacity (Taylor *et al*., 1996). Perhaps the parasite actively transports hemocyanin into the cell where it enters lysosomes. In the lysosome, it may react with oxidizers, forming reactive oxygen species, ultimately leading to lipofuscin formation.

Levels of lipofuscin evidently dissipated over time in cultured *Hematodinium*. Degranulation occurred very early in *Hematodinium* cultures, and lipofuscin was mostly depleted by the end of the 16 week trial. Although lipofuscin is presumably not degradable (Terman and Brunk, 2004), it would not seem beneficial for *Hematodinium* to accumulate large amounts of material that is indigestible. As lipofuscin-like granules only began degranulation after *Hematodinium* were taken from the host, perhaps they began to digest these granules to compensate for nutrients lacking in the medium, perhaps

the copper containing hemocyanin. This would indicate that *Hematodinium* is able to translate novel hydrolases, which are not found in vertebrates or invertebrates. However, another possible mechanism whereby lipofuscin-like granules were removed is through piecemeal degranulation and ultimately exocytosis of lipofuscin as occurs in mussels and humans (Fonseca *et al*., 2005; Zorita *et al*., 2006). Furthermore, bright orange/red globules were seen extracellularly in some Acridine Orange stained samples (Figure 2.21). Acridine Orange stains nuclear material green, but aggregates in acidic organelles such as lysosomes emits red or orange light when excited with UV light (Olsson, 1990). The extracellular material may be lipofuscin aggregates that were exocytosed. Alternatively, it could represent the dispersed contents of a lysed cell that was viewed adjacent to viable cells.

2.5 Conclusion

Six *in vitro* life stages of *Hematodinium* isolated from the Atlantic snow crab, *C. opilio* were observed (Figure 2.25). These include amoeboid trophont, coenocyte, sporont, dinospore, post-dinospore, and schizont stages. Many stages are similar to stages previously reported (Appleton and Vickerman, 1998; Li *et al*., 2011). There were some notable differences: writhing filamentous forms were not seen and amoeboid trophonts that were filamentous in morphology were observed. A distinction between amoeboid trophonts and filamentous trophont stages was not made. Multinucleated cell masses were termed coenocytes, as they were presumably the result of nuclear division without subsequent cytokinesis rather than the fusion of numerous cells. Clumps were observed in culture, and were senescent. Viable 'clump colonies' (Appleton and Vickerman, 1998) were not observed. Early sporonts and amoeboid trophonts were

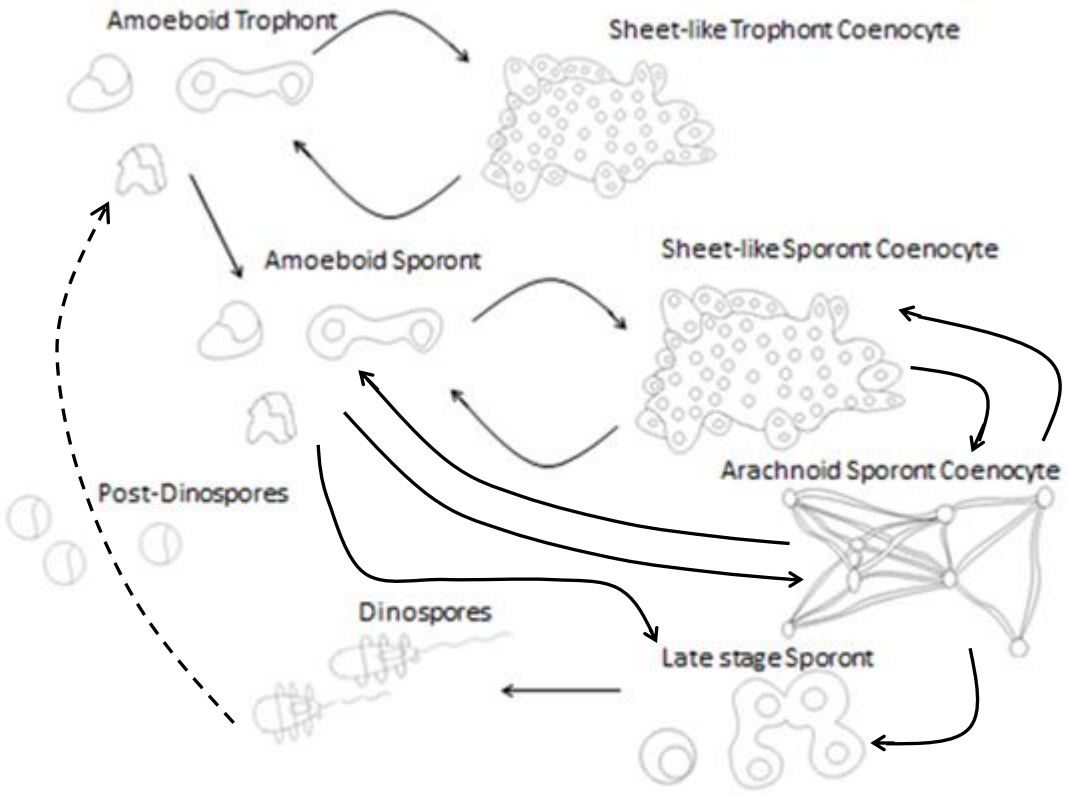

Figure 2.25: Life cycle diagram of *Hematodinium* isolated from *C. opilio* as viewed with light microscopy. Trophonts, the vegetative form of the parasite begin as amoeboid forms, but may transform to and revert from sheet-like coenocytes. Trophonts eventually transition to sporonts. Morphological differences between amoeboid trophonts and sporonts are absent. Amoeboid sporonts, like their trophont counterparts, may transition to and from sheet-like coenocytes. These coenocytes may also give rise to arachnoid coenocytes. These will late develop to late stage sporonts, where they will transition to dinospores. Dinospores eventually lose their flagella, becoming a post-dinospore form, and will likely become trophonts over time. The exact mechanism by which this is accomplished is unknown, thus the dotted arrow.

similar in morphology when viewed by light microscopy. Ultrastructural analysis is required to distinguish between these two stages.

Dinospores were observed after one month in culture and were also seen within dense aggregates of cells before motility was observed. Temperature and cell density likely play a role in dinospore motility and dispersal. These developed to a post-dinospore stage and did not transition to amoeboid trophonts (Appleton and Vickerman, 1998; Li *et al*., 2011). The medium may lack environmental and/or chemical cues necessary to successfully complete transition.

Hematodinium also possess filopodia-like structures that presumably attach to neighbouring cells. This may be important for the organism in cell to cell communication.

Notably, autofluorescent lipofuscin-like granules are present in all *Hematodinium*. It is likely that they form due to increased copper concentration during hemocyanin degradation within lysosomes. Although not tested in this study, measuring copper uptake by *Hematodinium* would be useful. These granules degranulated over time and likely leave the cell via exocytosis, or are digested. If digested, this would be unusual and would indicate that *Hematodinium* produces novel hydrolases.

This is the first investigation into the *in vitro* life stage development of *Hematodinium* isolated from *C. opilio* and is only the second *in vitro* investigation of *Hematodinium* to use ultrastructural techniques.

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Chapter 3: TRICHOCYST DEVELOPMENT IN *HEMATODINIUM* SP.

3.1 Introduction

Although trichocysts are commonly found in dinoflagellates, they have been studied more extensively in ciliates (Jakus, 1945; Yusa, 1963; Bouck and Sweeney, 1966; Bannister, 1972; Pollack, 1974; Hausmann, 1978; Miller *et al*., 2011). Trichocyst structure varies somewhat among species; it generally consists of a crystalline shaft within a vesicle, with the apical end of the vesicle attaching to the plasma membrane (Lee and Kugrens, 1992). Both ciliates and dinoflagellates apparently extrude their trichocysts in response to mechanical or chemical stimuli, although the exact nature of the stimulus has not been elucidated. The prevailing concept is that trichocysts serve a predatory or defensive function in protists (Harumoto and Miyake, 1991; Harumoto, 1994; Miyake and Harumoto, 1996; Tillmann and Reckermann, 2002; Zhang *et al*., 2011). Trichocyst discharge occurs explosively with rapid expansion of the crystalline shaft in most cases (Hausmann, 1978); some are internally discharged and held in sacs before exterior release (Messer and Ben-Shaul, 1969). The latter case may result from old or dying samples and therefore may not represent the typical release of trichocysts. The extrusion of trichocysts is apparently Ca^{2+} dependent, as calmodulin contributes up to 10% of the total mass of the trichocyst in *Paramecium* (Rauh and Nelson, 1981). Trichocysts could serve a role in osmoregulation as their discharge is mediated by a sudden influx of water

into the trichocysts, whereby a conformational change is induced, resulting in the striated appearance of discharged trichocysts (Jakus, 1945; Bouck and Sweeney, 1966). Trichocyst development in *Paramecium* sp. is regulated by a large multigene family encoding 100 genes, including three gene subfamilies that are each located on separate chromosomes. Furthermore, the gene products of one subfamily are simultaneously expressed (Madeddu *et al*., 1995). Although no studies have investigated genes involved in dinoflagellate trichocyst development, they are also likely composed of multigene families; dinoflagellates trichocysts are mechanistically and functionally similar to those of ciliates (Hausmann, 1978; Dodge and Greuet, 1987).

Although morphological similarities exist between trichocysts from dinoflagellates and ciliates and their functions are considered similar, there are notable differences. In *Paramecium*, the apical end of the trichocysts is electron dense and amorphous whereas in dinoflagellates, trichocysts have a complex system of fibres at their apical end that presumably attach to the cell membrane. The bases of ciliate trichocysts are broad and appear more conical in longitudinal sections than the much more uniform base of dinoflagellate trichocysts (for review, see Lee and Kugrens, 1992).

Dinoflagellates are photosynthetic or parasitic with many studies focused on photosynthetic dinoflagellates that are associated with toxic algal blooms (Steidinger and Baden, 1984; Granéli and Turner, 2006). Among 108 harmful micro algal species, ~ 72% are dinoflagellates (Møestrup, *et al*., 2009). Although parasitic forms are not known to release compounds toxic to humans, the parasitic dinoflagellate *Hematodinium* sp. is associated with disease in numerous economically significant crustaceans (Newman and Johnson, 1975; Maclean and Ruddell, 1978; Meyers *et al*., 1987; Latrouite *et al*., 1988;

Wilhelm and Boulo, 1988; Messick, 1994; Hudson and Lester, 1994; Hudson and Shields, 1994; Taylor and Khan, 1995; Wilhelm and Mialhe, 1996, Ryazanova *et al*., 2010).

The Atlantic snow crab (*Chionoecetes opilio*) is an economically important crustacean species to Atlantic Canada that has been affected by *Hematodinium* (Taylor and Khan, 1995; Dawe, *et al*., 2001; Mullowney *et al*., 2011). The life cycle of *Hematodinium* has not been described *in vivo*, and there are only two studies investigating its life cycle *in vitro* (Appleton and Vickerman, 1998; Li *et al*., 2011). Only one study has described *in vitro* life stages ultrastructurally (Appleton and Vickerman, 1998). One of the characteristics that are used to define the transition between trophont and sporont stages of the parasite is the development and appearance of trichocysts (Meyers, 1987; Appleton and Vickerman, 1998). There has been little research investigating trichocyst morphology and development in dinoflagellates in general, and there are no studies investigating trichocysts in parasitic dinoflagellates. As one of the principal characteristics used in defining *Hematodinium* life stages, trichocyst development was explored ultrastructurally in *Hematodinium* isolated from fresh hemolymph of *C. opilio* and from *Hematodinium* cultured *in vitro*. In the present study, trichocyst development was characterized and it was demonstrated that trichocysts from *Hematodinium* resembled those from other photosynthetic dinoflagellates, with slight differences in morphology.

3.2 Materials and Methods

3.2.1 Collection of Atlantic snow crabs

Atlantic snow crabs (*C. opilio*) were collected during annual population assessment surveys in White Bay and Notre Dame Bay, Newfoundland during September 2010 and 2011. Crabs obtained in 2010 were caught using pot traps (135 mm and 25 mm mesh size) on the grounds of strata 610 and 611 (200-400 m in depth), and those caught in 2011 were caught in a similar manner in stratum 614 (301-400 m in depth). Water temperatures for stratum 614 normally ranged between 0-1°C in early fall, and strata 610 and 611 between -1°C and 3°C (Mullowney *et al*., 2011). Crabs were kept in coolers layered between seawater soaked burlap, and placed above saltwater ice until reaching shore. On shore, coolers were transported to St. John's, Newfoundland, where they were shipped via air cargo to the Atlantic Veterinary College (AVC), University of Prince Edward Island. The time between snow crab harvest and their arrival at AVC was usually 24-48 h. Nine crabs showed morphological changes consistent with Bitter Crab Disease (BCD) (one from 2010, eight from 2011). These changes included discoloured or opaque cuticle and milky white hemolymph.

Additionally, two Atlantic snow crabs showing morphological changes consistent with BCD were caught by Campelen shrimp trawling off the coast of Nova Scotia during October 2010 and September 2011 at positions 44° 34.86N, 58° 40.29W and 44° 23.23N, 63° 28.11W respectively. The water depth was ~ 100 m and bottom temperatures ranged between 0-3°C at these locations (Tremblay, 1997). These crabs were kept in coolers packed with saltwater ice and transported by land to the AVC, University of Prince Edward Island. The time between snow crab harvest and their arrival at AVC was usually 24-48 h.

3.2.2 Necropsy of Chionoecetes opilio and hemolymph collection

Upon arrival, crabs were placed in an aerated holding tank at 0°C with 33-34 ppt salinity for 1-4 h to recover from the stress of shipment. Ice packs were placed in the holding tank in an attempt to keep water temperature at 0°C. Crab viability was assessed by observing mandible or limb movement and response to 70% ethanol (EtOH) sprayed on the mouth and gills. Only live crabs were used in the isolation of dinoflagellates. Carapace width, sex, hemolymph refractive index, and any gross lesions were recorded, and crabs were exsanguinated. Hemolymph was collected from the base of the first periopod using a 3 ml or 5 ml syringe with a 22 gauge needle. The arthrodial membrane was surface sterilized with 70% EtOH prior to exsanguination. Hemolymph (200 µl) was placed into a single well of a 96 well plate containing 800 µl 80% ethanol for future DNA extraction and genotype confirmation by polymerase chain reaction (as part of a separate study). Remaining hemolymph was stored on ice until the completion of all necropsies. After exsanguination, the carapace of the crab was removed, and the heart, hepatopancreas, eyestalk, gills, gonad, gut, and leg muscle were surgically removed and placed in Davidson's seawater fixative for future histopathological examination (as part of Dr. Melanie Buote's Ph.D. research). Necropsies usually took 3-4 h, depending on the number of crabs obtained in each shipment.

3.2.3 Initiation and maintenance of cell cultures

After necropsy, hemolymph containing suspect *Hematodinium* from BCD evident crabs was cultured *in vitro*. Syringes containing hemolymph were gently agitated to ensure adequate mixing, and hemolymph was transferred to 1.7 ml microcentrifuge tubes within a Class II biological safety cabinet (Sterigard® III Advance, The Baker Company). Infected hemolymph (40 µl) was added to 1.94 ml of *Nephrops* medium (See

Appendix A) in 24 multiwell plates (Falcon) and incubated in an incubator (Binder-APT.line™ KB) at 0 ± 0.2°C. Cultures were viewed on an inverted phase contrast microscope (Nikon) with transmitted light, every two to three days, to monitor growth and for contaminating organisms. If cultures became too dense (cells covered ~2/3 of culture well), they were diluted 1:1 with fresh medium. If cultures developed contaminating organisms, a 1 ml aliquot of sample was sent to AVC Diagnostic Services, where the organism was isolated, classified, and tested against a series of antibiotics to elucidate effective treatment options. Culture medium was changed every 7-10 days using the following procedure: cultures were resuspended by gentle pipetting and a 1 ml aliquot was transferred into 1.7 ml microcentrifuge tubes, and centrifuged at 900 X g for 5 min at 4°C; care was taken to minimize disruption of cells that were attached to plate surface. Supernatant was discarded, and the cell pellet was resuspended in 1 ml of fresh *Nephrops* medium. The fresh suspension was transferred gently by pipette into its original culture well, which had been kept at 0 ± 0.2°C during centrifugation.

3.2.4 Preparation of samples for transmission electron microscopy

Hemolymph from BCD suspect crabs was fixed for transmission electron microscopy (TEM) the same day as necropsies were conducted, in all cases. In addition, samples from *in vitro* culture were fixed and processed either one or two weeks following initial hemolymph collection and every two weeks subsequently. Cultures were terminated after 16 weeks. Only cultures from 2011 were fixed and processed in this manner; cultures from 2010 lacked sufficient material to complete a similar timeline.

Crab hemolymph from 2010 was fixed for TEM: hemolymph (~75 µl) was added to 2 ml of 3% glutaraldehyde (Canemco) in artificial sea water (ASW; Instant Ocean) (33

ppt) and stored at 0°C for either 12 days (Newfoundland sample) or 24 h (Nova Scotia sample). Samples were then washed twice in ASW and post-fixed in 1% OsO_4 in ASW for 30 min. Prior to washing, samples were centrifuged at 1700 X g at room temperature (RT; 20-22^{o}C) for 5 min. During washing, samples were centrifuged at 3800 X g at RT for 10 min. After post-fixation, samples were centrifuged at 6700 X g for 10 min at RT. Osmium tetroxide solution was removed from samples, and the resulting pellet was overlaid with 4% agar (Fisher). Samples were incubated at 4°C for 10 min to solidify agar. Samples were removed from the microcentrifuge tube, cut into small pieces with razor blades, and placed in glass vials containing 50% ethanol (EtOH). Samples were dehydrated using a graded series of EtOH washes at concentrations of 50%, 70%, 95%, and 100%, then cleared in propylene oxide (PO). Each wash was performed twice for 10 min each with the exception of the first step, where only one wash was performed. After clearing in PO, samples were infiltrated with Epon resin by placing them in a mixture of Epon/PO at a ratio of 1:1 for 1 h, at a ratio of 3:1 for 2 h, and then in 100% Epon overnight. Epon resin was made as described in Appendix D. After infiltration, samples were placed in BEEM capsules (Canemco) filled with Epon, and incubated at 60^{o}C overnight to polymerize the Epon.

Crab hemolymph from 2011 (150 µl/sample) was fixed for TEM in 2 ml of 3% glutaraldehyde in ASW overnight at 4°C. Samples were washed twice in ASW for 5 min at RT. Prior to washing, samples were centrifuged at 1700 X g at room temperature RT for 5 min. During washing, samples were centrifuged at 1700 X g at RT for 5 min. Samples were post-fixed in 1% OsO_4 in ASW for 30 min, and a pellet was obtained by centrifuging samples at 1700 X g at RT. Osmium tetroxide was removed from above the

pelleted samples and then the samples were overlaid with agar, dehydrated, infiltrated, and placed in BEEM capsules, and polymerized as described above.

Cultures from 2011 crabs were fixed as follows: one millilitre of 6% glutaraldehyde in *Nephrops* saline at pH 7.6 was added to equal amounts of culture and mixed thoroughly by pipetting. *Nephrops* saline differs from *Nephrop*s medium; it does not contain antibiotics or foetal bovine serum. Samples were washed, post-fixed, overlaid in agar, dehydrated, infiltrated with Epon and polymerized as described for initial hemolymph samples obtained from crabs caught in 2011.

3.2.5 Sectioning and imaging of samples for transmission electron microscopy

Thick sections (0.5 μm) were cut with glass knives (Leica) on an ultramicrotome (Reichert-Jung, Ultracut E, Leica Microsystems), mounted on glass slides, stained with 1% toluidine blue in 1% sodium tetraborate solution and viewed with a light microscope to select areas for ultrathin sectioning.

Ultrathin sections (90 nm) were cut using a diamond knife (Pelco) on an ultramicrotome (Reichert-Jung, Ultracut E, Leica Microsystems). Sections were collected onto 200 mesh copper SuperGrids™ (SPI Supplies) and stained with uranyl acetate (saturated aqueous solution) for 30 min at RT, and then stained with lead acetate for 2 min at RT as described previously (Sato, 1968). After each staining period, samples were triple washed in dH_2O for 30 sec. Grids were viewed with a transmission electron microscope (Hitachi H7500) operated at 80 kV and digital images were taken using an AMP XR40 side mounted camera.

3.2.6 Trichocyst measurements

All measurements were performed using FIJI image program (FIJI is a freeware program based on Image J and can be downloaded at http://fiji.sc), and descriptive statistics (mean, standard error, minimum and maximum values) were obtained using Minitab 15 Statistical Software. Sixteen primordial vesicles in nine cells were measured. To determine the diameter of a particular primordial vesicle, two perpendicular measurements were taken of the primordial vesicle, and the mean calculated. Trichocyst core dimensions were measured from 100 mature trichocysts in 18 different cells. As mature trichocyst cores were square in cross section, only one measurement from each core was obtained. Measurement of dimensions within the crystalline lattice was performed on three trichocysts. A total of 39 striations within crystalline cores were measured. Dimensions of striations from degraded trichocysts were also measured and analyzed. Fifty striations from six degraded trichocysts were measured.

3.3 Results

3.3.1 Structure and cell localization of mature trichocysts

Hematodinium from 2010 hemolymph samples had fully developed trichocysts. In the 2011 hemolymph samples, some of the *Hematodinium* contained mature trichocysts from the onset, while others developed trichocysts in culture (See Chapter 2), and some did not develop trichocysts during the entire duration of the experiment. *Hematodinium* trichocysts from Newfoundland and Nova Scotia appeared morphologically identical.

Fully developed or mature trichocysts appeared as long, rectangular shaped organelles, bounded by a single membrane. The body of the trichocyst contained a crystalline core that appeared rigid, whereas the apical region appeared flexible (Figure 3.1). Cross sections through the trichocyst body revealed an elaborate square crystalline

core with striations every 10.2 ± 0.3 nm (mean ± standard error, n=3) forming a lattice or grid pattern. The crystalline core was 223 ± 2.11 nm wide (range 142-275 nm (n=100) (Figure 3.2). The apical region was where the trichocyst core ended and the trichocyst membrane began to taper. The apical region was complex and tapered, containing various structures and appeared to dilate slightly near the inner amphiesmal vesicle

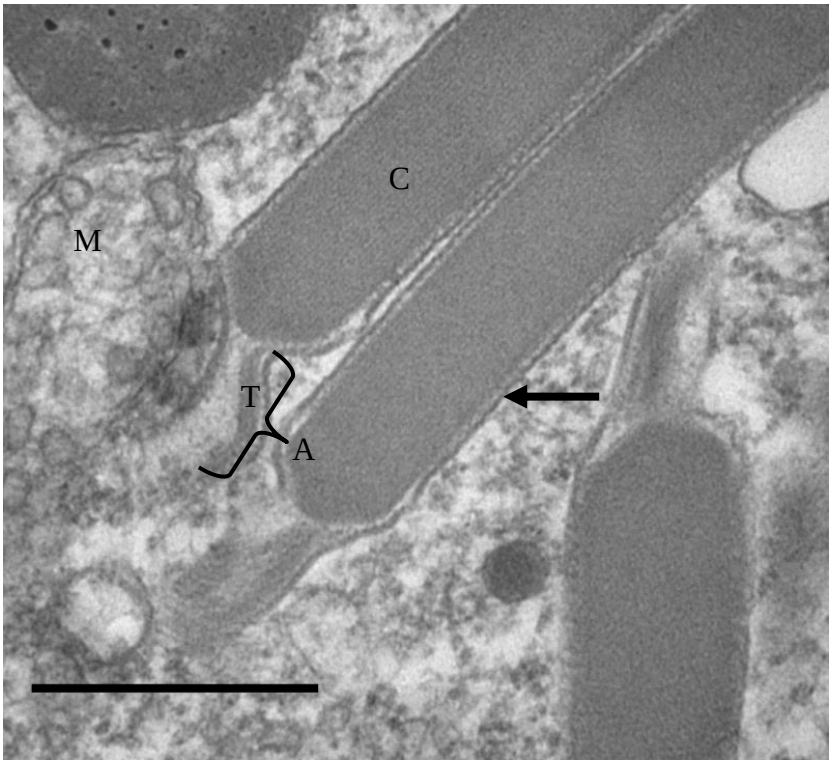

Figure 3.1: Longitudinal sections through portions of mature *Hematodinium* trichocysts. Note the single membrane (arrow) surrounding the electron dense trichocyst core (C) and the apical region (A). The apical region contains is flexible, tapers, and contains tubules (T) that attach to the tip of the core. M= mitochondrion. Scale bar: 500 nm.

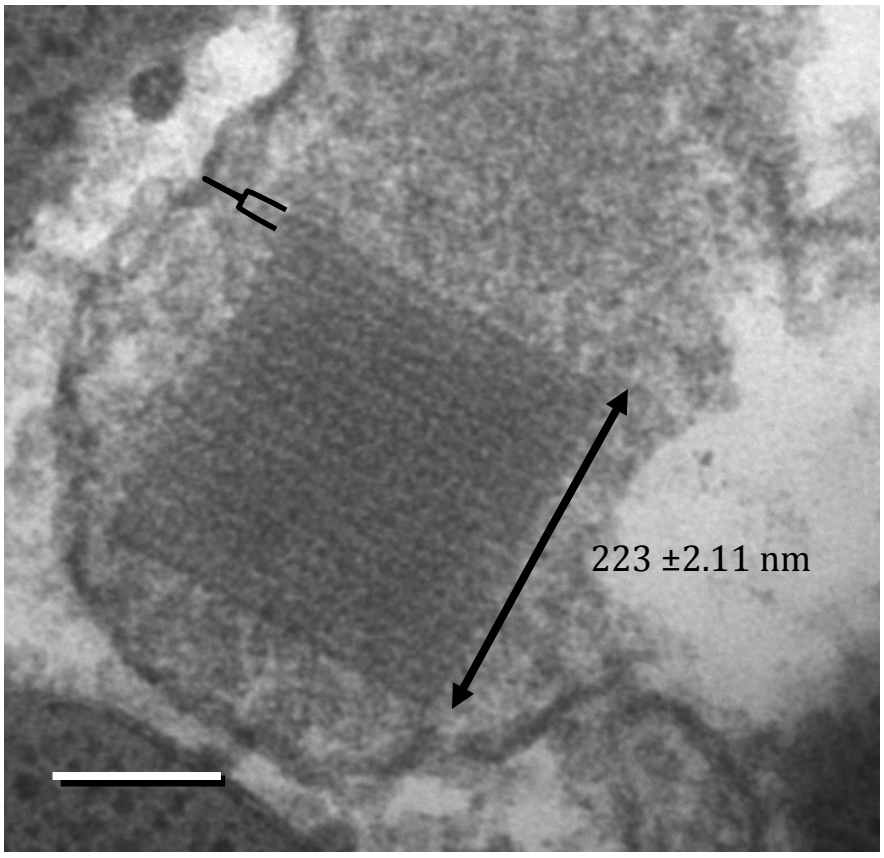

Figure 3.2: The *Hematodinium* trichocyst core was square in cross section with the width of the core measuring ~ 223 ± 2.11 nm (n=100). The core was a crystalline lattice structure with striations appearing at regular 10.2 ± 0.3 nm (n=3) intervals (fork). Scale bar: 100 nm.

membrane (Figure 3.3). Although it was unclear whether the membrane of the mature trichocyst was fused with the amphiesmal vesicle membrane, there was close association between the trichocyst membrane at the apical end and the inner amphiesmal vesicle membrane, and cell membrane in some regions (Figure 3.3). Furthermore, near the tip of the apical end, there was often a semi-electron dense region lining the periphery of the membrane and circular structure in the centre (Figure 3.3). An elaborate array of tubules was arranged around the periphery of the trichocyst membrane in the apical region (Figure 3.3; Figure 3.4). A maximum of thirteen tubules was observed in a single trichocyst; tubules that were cut in cross section were ~15 nm in diameter. These tubules presumably attached to small cuboidal appendages on the apex of the trichocyst core (Figure 3.3). Furthermore, the inner surface of the trichocyst membrane in the apical region was decorated with electron dense material arranged in hoops around the periphery (Figure 3.3).

In numerous cells, there was obvious clustering of trichocysts to a particular cellular location (Figure 3.5, Panel A); however, trichocysts in some parasites appeared to be distributed ubiquitously in various orientations (Figure 3.5, Panel B).

3.3.2 Trichocyst development

Various stages of trichocyst development were observed within different *Hematodinium* cells and within individual *Hematodinium*. Primordial vesicles measuring approximately 519 ± 40.2 nm (n=16) in diameter with a maximum size of 856 nm were

observed (Figure 3.6). Granular material within the primordial vesicle was relatively homogenous and the surrounding membrane had a corrugated appearance. Primordial vesicles were often associated with the trans-face of the Golgi apparatus. Some

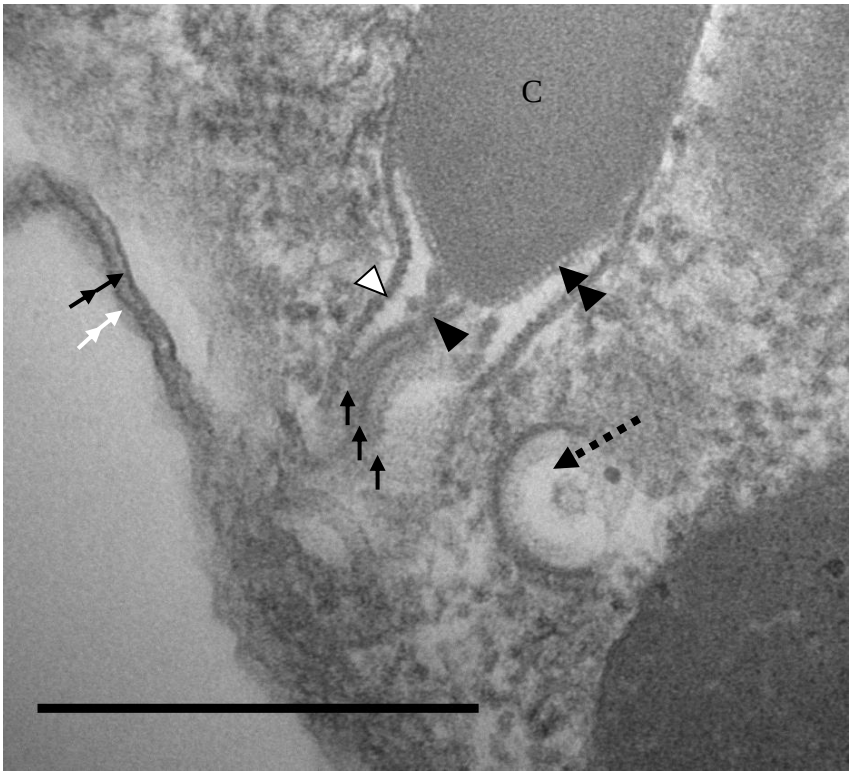

Figure 3.3: Apical region (and possible attachment site) of mature *Hematodinium* trichocysts. The trichocyst core (C) formed a conical shape at its apical end. Trichocyst tubules (black arrowhead) attached to small cuboidal appendages (double black arrowhead) that covered the apex of the core. Electron dense hoops (black arrows) encircled the inner surface of the apical trichocyst membrane and appeared attached to electron dense decorations (white arrowhead) along the inner portion of the trichocyst membrane in cross section. The distal portion of an apical region was seen in cross section in an adjacent trichocyst (dashed arrow). Note the material along the periphery

extending from the electron dense apical membrane formed a hoop as well as material in the center of the tip region. The tip of the apical region approached the plasma membrane (double black arrow) and possibly the inner amphiesmal vesicle membrane (double white arrow): Scale bar: 500 nm.

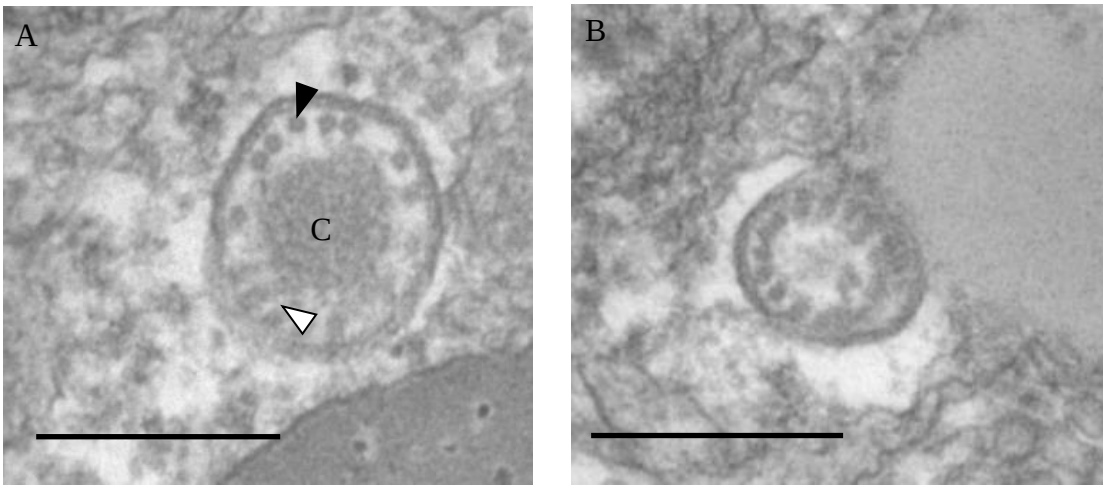

Figure 3.4: Cross sections through the apical region of mature *Hematodinium* trichocysts: A) the tip of the conical portion of the trichocyst core is shown (C). Note the peripheral distribution of electron dense tubules that appeared in cross section (black arrowhead) and tangential section (white arrowhead). B) The cross section was likely through a portion of the trichocyst that was closer to the cell membrane attachment site. The trichocyst core had tapered and most tubules appeared tangentially cut. Scale bar: 250 nm.

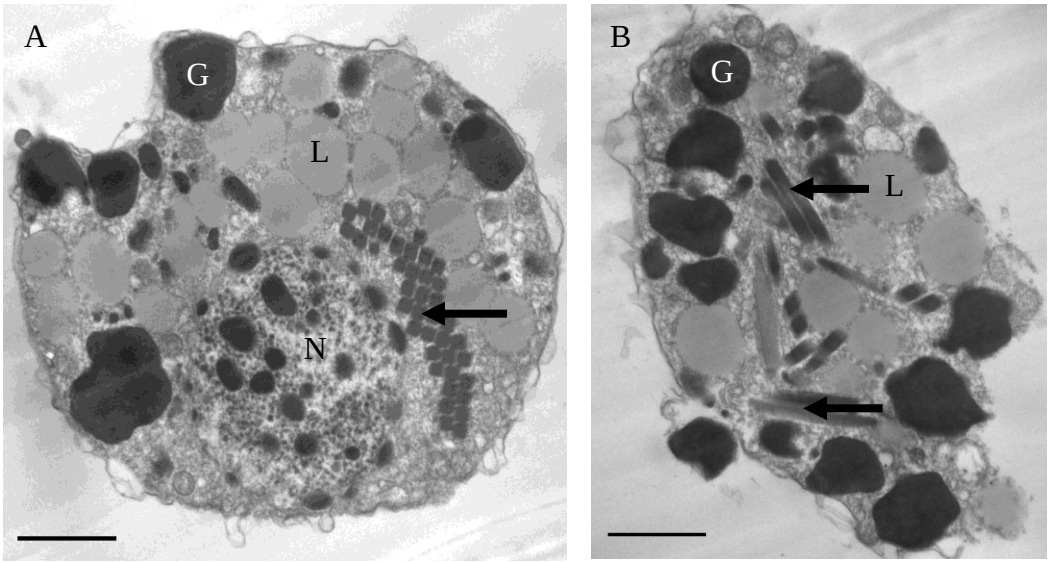

Figure 3.5: Arrangement of trichocysts within *Hematodinium.* A). Trichocysts (arrow) often appeared in clusters near the nucleus (N). B) Trichocysts (arrows) were also found in various orientations within *Hematodinium*. G= granular vesicle L= lipid body. Scale bar: 2 μm.

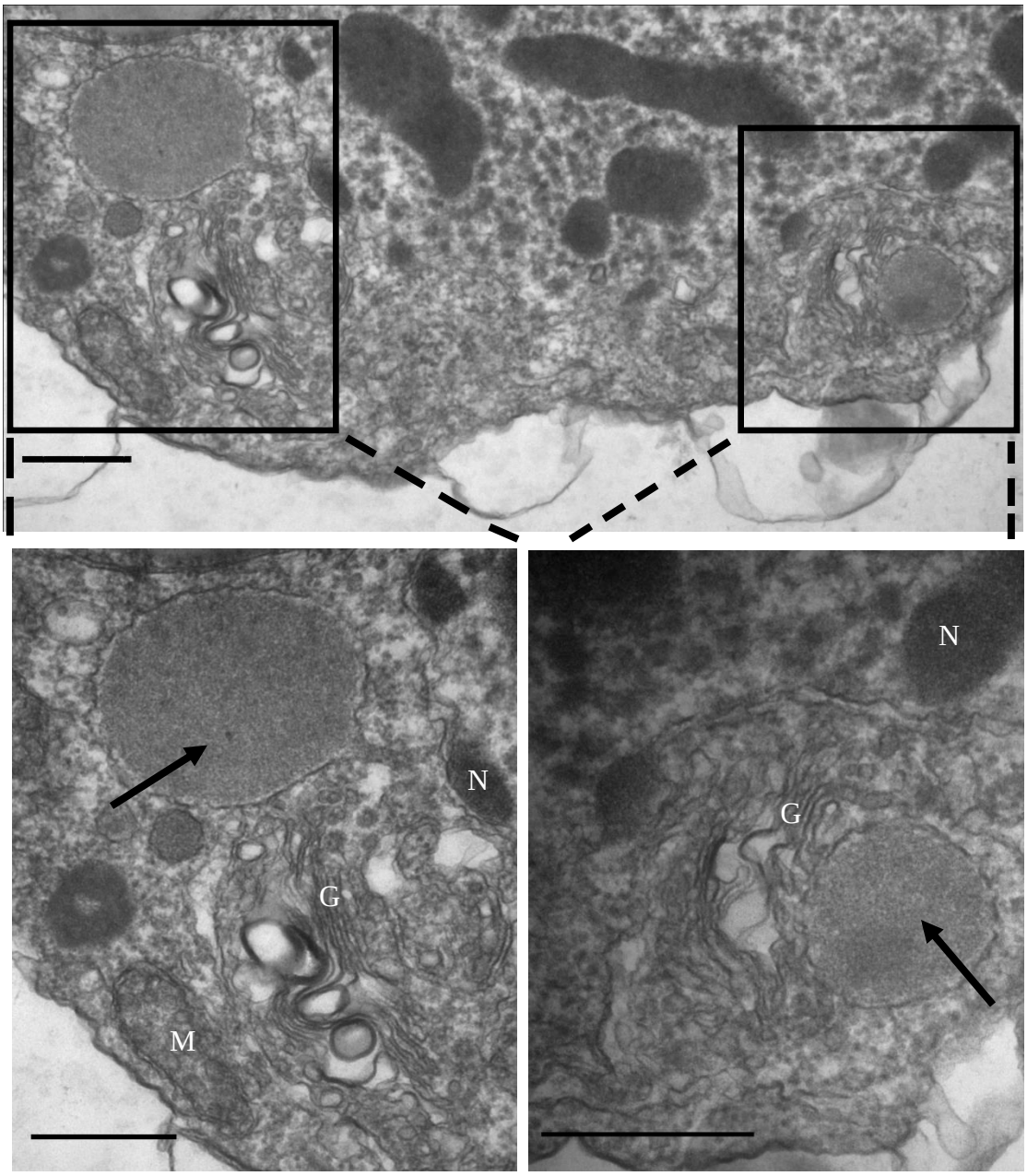

Figure 3.6: Primordial trichocysts of *Hematodinium* appeared as large (519 ± 40.2 nm; n=16) vesicles containing granular material (arrow) associated with the trans-face of the Golgi apparatus (G). The vesicles were enclosed by a single membrane that has a wavy or corrugated appearance. N= nucleus; M= mitochondria. Scale bar: 500 nm.

primordial vesicles were also seen within clusters of mature and developing trichocysts (Figure 3.7). Developing trichocysts possessed similar, granular material to primordial vesicles; during development, condensation of granular material occurred resulting in an electron dense core within the more amorphous primordial granular material. Granular material surrounding the electron dense core condensed during trichocyst maturation, leaving an electron lucent area between the trichocyst membrane and the core (Figure 3.7). In the final stages of development the membrane wrapped tightly around the core, leaving little space between the core and membrane (Figure 3.7). Trichocysts were deemed mature after the membrane was closely apposed to the core.

3.3.3 Trichocyst degradation

In *Hematodinium* that had transformed from dinospores to post-dinospores, trichocysts appeared to be undergoing macro-autophagy. After ~2 weeks, dinospores lost their flagella and became spherical in morphology. They also showed unusual nuclear characteristics, and were thus termed post-dinospores. Although these cells seemed to be viable, as they actively took up Neutral Red vital stain, they showed numerous phagolysosomes and could have been moribund. (They are discussed in greater detail in Chapter 2). Trichocysts were seen in large secondary lysosomes along with other granular/cellular material (Figure 3.8). Striations measuring 13.8 ± 0.23 nm (n=6) were clearly seen in these degraded trichocysts cores when viewed in longitudinal sections, unlike other trichocysts.

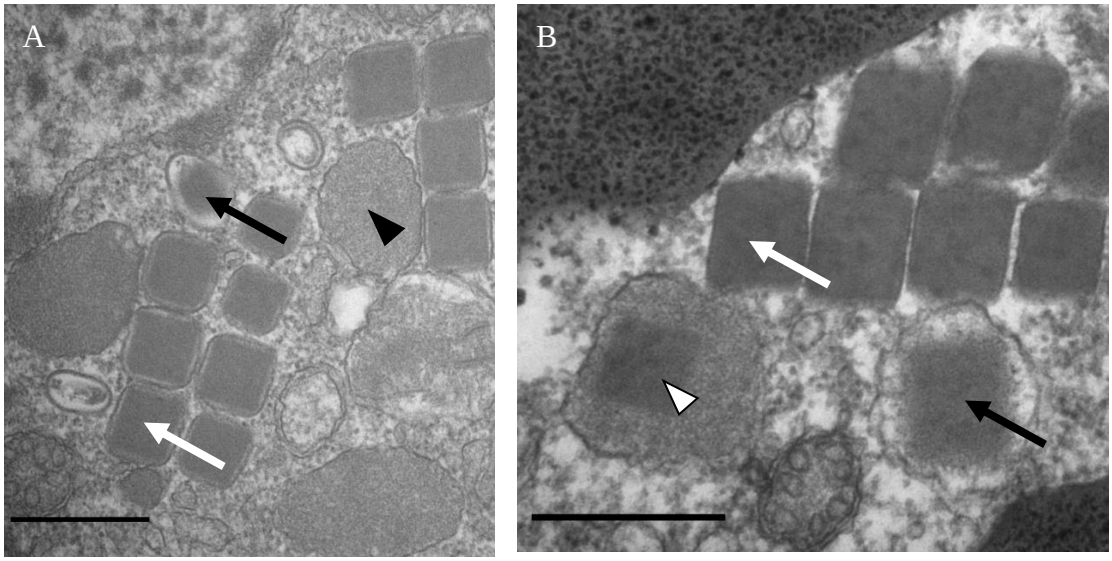

Figure 3.7: Mature and developing trichocysts were observed in individual *Hematodinium*. A) Primordia (black arrowhead) were seen within a cluster of mature and developing trichocysts. Mature trichocysts (white arrow) had a single membrane tightly surrounding the electron dense trichocyst core. In the developing trichocyst (black arrow), the granular material had condensed leaving an electron lucent area separating it from the membrane. B) Two trichocysts at different stages of development were clustered with mature trichocysts (white arrow). The trichocyst earlier in development (white arrowhead) showed an electron dense trichocyst core forming with granular material and a single membrane having a corrugated appearance. The trichocyst later in development (black arrow) had an electron dense trichocyst core, little granular material, and an electron lucent area between the core and membrane. Scale bar: 500 nm.

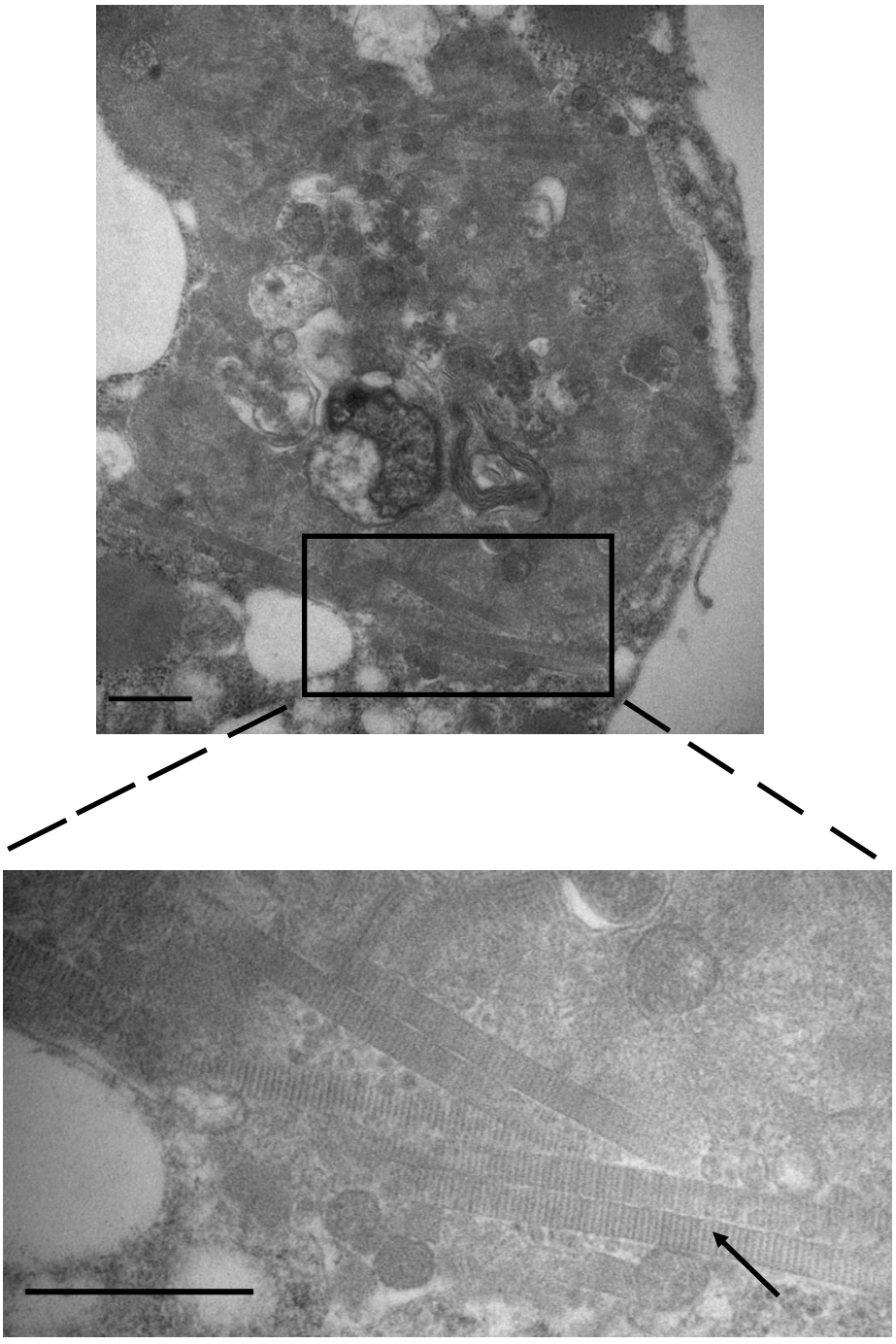

Figure 3.8: Degenerated *Hematodinium* trichocysts (arrow) were evident within the contents of a secondary lysosome. Trichocysts remained as elongated, rod-like structures. Distance between striations of degenerated trichocysts were 13.8 ± 0.23 nm (n=6). Scale bar: 500 nm.

3.4 Discussion

3.4.1 Ontogeny of trichocysts

Hematodinium sp. is a parasitic dinoflagellate of numerous crustacean species worldwide (Newman and Johnson, 1975; Maclean and Ruddell, 1978; Messick, 1994; Meyers *et al*., 1987; Latrouite *et al*., 1988; Wilhelm and Boulo, 1988; Hudson and Lester, 1994; Hudson and Shields, 1994; Taylor and Khan, 1995; Wilhelm and Mialhe, 1996; Ryazanova *et al*., 2010). To date, *Hematodinium*'s life cycle has not been characterized *in vivo*; however, it has been isolated from two hosts (*Nephrops norvegicus* and *Callinectes sapidus*) and the life cycle explored *in vitro* (Appleton and Vickerman, 1998; Li *et al*., 2011). The principal criterion to define the non-vegetative stage of the parasite (sporont) from the vegetative stage of the parasite (trophont) is the appearance of trichocysts (Appleton, 1996). Thus, knowledge of trichocyst development in *Hematodinium* is paramount to understanding sporont development. Interestingly, Seo and Fritz (2002) note that vegetative cysts of *Pyrocystis lunula* and *P. noctiluca* have trichocysts, unlike the vegetative stages of *Hematodinium*; thus, trichocysts may be an important characteristic in sporont development in *Hematodinium*, but not in other dinoflagellates; however, more research is required. There is little knowledge of trichocysts in dinoflagellates, (much of our knowledge of trichocysts has been drawn from studies in ciliates), and there are no reported studies of trichocyst development in *Hematodinium*.

Crustaceans showing clinical signs of BCD may harbour either trophont or sporont stages of the parasite; trichocysts were not present in all *Hematodinium* collected from infected crab hemolymph. Furthermore, all late-stage infected crustaceans and

hemolymph samples appeared grossly similar; there are no easily discernible differences between crustaceans infected with sporonts or trophonts, nor are trophont and sporont stages distinguishable using light microscopy.

Trichocysts do not develop synchronously among *Hematodinium* isolated from a single crab, or within the same parasite. This was initially surprising; however, trichocyst development in *Hematodinium* likely takes days to weeks. Trichocysts from *Paramecium* are the result of ~100 gene products (Madeddu *et al*., 1995), and the cell likely requires significant energy reserves for this developmental process. Although the numerous lipid droplets found within *Hematodinium* cells aid in providing nutrients during periods of high energy consumption, *Hematodinium* would still benefit from producing trichocysts incrementally as this would enable the cell to maintain necessary resources for normal biological functions during production. Although the genome size of dinoflagellates varies, Tang (1996) hypothesized that the relatively large genome size of dinoflagellates requires considerable energy to maintain normal function. Building trichocysts incrementally therefore would allow the cell to discontinue production should there be a shortage of nutrients.

Ultrastructural examination revealed vesicles measuring 519 ± 40.2 nm (n=16) containing granular material surrounded by a single membrane with a corrugated appearance. These vesicles were often in association with the Golgi apparatus, and with other trichocysts. These are thought to be primordial vesicles of early, developing trichocysts as described in other dinoflagellates and ciliates (Yusa 1963, 1965; Bouck and Sweeney, 1966). Other vesicles contained varying amounts of granular materials and a distinct crystalline core in later stages of developing trichocysts.

Trichocyst specific proteins synthesized in the rough endoplasmic reticulum travel to the Golgi where they likely accumulate in a vesicle at the trans-face. This primordial vesicle grows in size from the addition of newly synthesized proteins. It likely disassociates from the Golgi after reaching a certain volume and travels to its final cellular destination among developing and mature trichocysts. It then begins to polymerize the complex structure of the crystalline core. How the organism regulates this is unknown. Fusion of other vesicles to/with a developing trichocyst have not been observed to date, indicating that developing trichocysts do not receive additional materials through packets of proteins stored in other vesicles. Apparently, all necessary components for trichocyst assembly are located in the primordial vesicle. This is further supported by the observation of an early trichocyst that had begun to lengthen and develop tubules near its apical end, even before crystalline core formation. Furthermore, based on the appearance of primordia adjacent to mature and developing trichocysts, proteins necessary for crystalline core assembly likely polymerized within primordia. As they polymerized, the primordial vesicle also appeared to change shape, becoming elongated. The granular material likely condensed, leaving an electron lucent area between the membrane and crystalline core. Finally, the membrane tightly surrounded the crystalline core, removing the electron lucent area. This is different than that described in other marine dinoflagellates (Bouck and Sweeney, 1966; Schmitter, 1971) where the membrane remains circular and there is a relatively large electron lucent area between the crystalline core and membrane. These marine dinoflagellates are photosynthetic, unlike *Hematodinium*, which may explain the apparent differences between trichocysts.

3.4.2 Structure of mature trichocysts

Mature trichocysts are composed of two areas, the body and the apical region. The body consists of the electron dense crystalline core which is tightly surrounded by a single membrane. The core is long and rectangular shaped, composed of a proteinaceous crystalline lattice (Madeddu *et al*., 1995). The apical region begins where the core (and trichocyst membrane) begins to taper. Within the apical region, up to thirteen tubules measuring ~15 nm in diameter line the periphery and attach to cuboidal appendages covering the apex of the trichocyst core. As the apical region tapers, tubules draw closer and attach part way along the apical region. The structure that they attach is unknown. The tip/apex of the apical region widens slightly and contains electron dense regions along the periphery and centre. The tip is closely associated with the plasma membrane, but fusion to the plasma membrane while in its resting state is unknown. Additionally, electron dense hoops line the inner surface of the trichocyst membrane in the apical region and are seen in cross section as electron dense decorations of the membrane. A schematic representation of a mature *Hematodinium* trichocyst is depicted in Figure 3.9.

The core of the trichocyst is a proteinaceous crystalline structure. Although several proteins from ciliate trichocysts have been characterized (Madeddu *et al*., 1995), little is known about the composition of the dinoflagellate trichocyst, but is believed to be similar to that of ciliates. The core is a rigid crystalline structure, whereas the apical region, which lacks the crystalline core, appears flexible. Trichocysts appear somewhat flexible and are likely capable of tolerating some mechanical stress. Although some speculate that trichocysts act as pseudo-osmoregulators in dinoflagellates and are discharged by an influx of water when they are in an hypoosmotic environment (Bouck

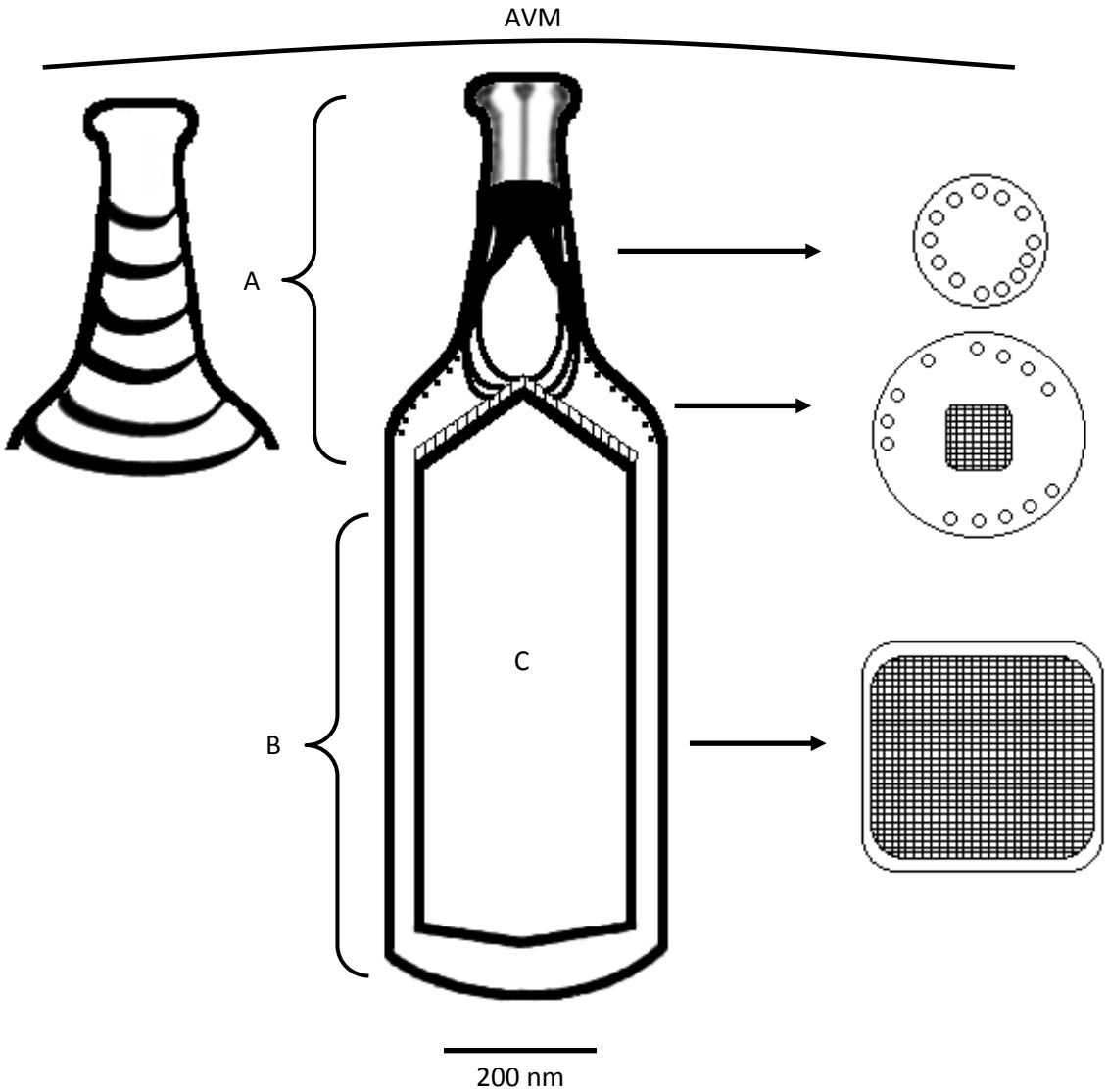

Figure 3.9: Schematic diagram of mature trichocyst from *Hematodinium*. A three dimensional representation of the internal surface of the apical end is shown on the left and a longitudinal section in the middle. Cross sections of the trichocyst are depicted on the right. There are two main regions of the trichocyst, the body (B) and the apical end (A). The body is composed of a crystalline core (C) which is tightly surrounded by a single membrane. In the apical end, the crystalline core and trichocyst membrane taper. Covering the apex of the crystalline core are small cuboidal appendages to which tubules attach. Tubules arrange in an ordered fashion along the periphery of the trichocyst apex, and attach part way along the apical end. The tip of the apical end widens, and is closely associated with the inner amphiesmal vesicle membrane (AVM). Additionally, electron dense decorations line the periphery of the trichocyst. Hoops surround the inner surface of the trichocyst (left image) and attach to the electron dense decorations.

and Sweeney, 1966; Yusa 1963, 1965), trichocyst expulsion in *Paramecium* can be induced both chemically and mechanically (Hausmann, 1978; Adouette, 1988). The parasite appears limited to spaces and cavities bathed in hemolymph and thus subject to frequent or constant circulation (Newman and Johnson, 1975; MacLean and Ruddell, 1978; Meyers *et al*., 1987; Field *et al*., 1992; Love *et al*., 1993; Hudson and Shields, 1994; Messick, 1994; Shields, 1994; Field and Appleton, 1995; Taylor and Khan, 1995; Wilhelm and Mialhe, 1996; Taylor *et al*., 1996; Shields and Squyars, 2000; Stentiford *et al*., 2002), as neither trophonts nor sporonts appear capable of perforating membranes and epithelia of organs and tissues (Morado, personal communication). Although crustaceans have an open circulatory system and have relatively low hemolymph pressure (mean = 1.9 kPa in *Cancer magister*), their stroke volume, heart rate, and hemolymph distribution may vary depending on activity (McMahon and Burnett, 1990). Sustained pressures up to 6 kPa have been observed in lobsters (Belman, 1975). Thus, one can envision a scenario whereby *Hematodinium*, free in circulation, may collide with neighbouring cells, hemocytes and vessels. If discharge is mechanically induced, it would be essential that trichocysts be able to absorb impact forces that it is exposed to regularly, to prevent premature expulsion.

Hoops lining the internal surface of trichocysts observed in the apical region were similar to those described by Bouck and Sweeney (1966) for other marine dinoflagellates. Hoops were not observed along the body of the trichocyst. The electron density of the trichocyst core and the close association between the trichocyst membrane and the core would render the viewing of such hoops in this region difficult.

We show evidence, for the first time, of tubules in the apical region of the trichocyst of *Hematodinium*. Previous studies have described electron dense fibres at the apical end of trichocysts (Bouck and Sweeney, 1966; Hausmann, 1978; Margulis *et al*., 1993). The arrangement of tubules differs from trichocyst fibres of other protists in that tubules are arranged near the periphery of the trichocyst. Furthermore, very small cuboidal appendages cover the apex of the crystalline core, which is a novel observation. Tubules do not attach at the tip of the trichocyst but instead attach part way along the apical region. The tip of the apical region broadens and contains electron dense regions lining the periphery and centre. These electron dense regions are not well understood. The electron density along the periphery (as seen in Figure 3.3) could be where hoops attach to the membrane, which would indicate that there is considerable depth to hoops. It would not explain, however, the observed electron density of the centre. Although the tip is in close association with the inner amphiesmal vesicle membrane, direct fusion of the trichocyst to this membrane while in its resting state was not discernible. Due to its position in the cell, the tip of the apical region likely plays a major role in trichocyst fusion to the amphiesmal membranes and possibly to the plasma membrane, and the ultimate expulsion of the trichocyst.

3.4.3 Trichocyst degradation

In post-dinospore *Hematodinium*, trichocysts appeared to be undergoing macro-autophagy. Apparent remains of trichocyst cores are seen within secondary lysosomes along with other cellular material, and appeared similar to discharged trichocysts (Bouck and Sweeney, 1966; Messer and Ben-Shaul, 1969). Trichocysts in ciliates occur in autophagocytic vesicles (Landers, 1991), but do not appear as long striated forms. Others

have described long, striated trichocysts within vacuoles, similar to what we observed, and have described them as trichocyst sacs (Messer and Ben-Shaul, 1969). Unlike our observations, however, trichocyst sacs, as described by Messer and Ben-Shaul (1969), appear to be released from the cell similar to secretory granules. We did not observe the release or expulsion of trichocysts during our studies.

3.4.4 Possible roles for trichocysts in parasite transmission

Filamentous trophonts and early arachnoid life stages of *Hematodinium* have an affinity towards the hepatopancreas of the Norway lobster, *Nephrops norvegicus* (Field and Appleton, 1996; Appleton and Vickerman, 1998; Stentiford *et al*., 2001). Furthermore, *Hematodinium* apparently attaches to the basal lamina of the hepatopancreas in *N. norvegicus* and the wall of hemolymph vessels in the harbour crab, *Liocarcinus depurator* (Stentiford *et al*., 2003; Small *et al*., 2012). Apparent attachment may occur through multiple mechanisms. Attachment to host organs could occur through various protein-protein interactions at the host-parasite interface. Alternatively, *Hematodinium* may not be attached at all, but simply aggregating along the basal lamina, giving the appearance of attachment when viewed for histology. However, trichocysts may play a role in parasite attachment and ultimate development.

Dinospores are presumably the infective life stage of the *Hematodinium* parasite (Appleton and Vickerman, 1998; Stentiford and Shields, 2005). Based on our current understanding of the *Hematodinium* life cycle *in vitro*, filamentous trophonts and arachnoid sporonts are successors of dinospores. Thus, upon entry into the suitable host, the dinospore may attach to the hepatopancreas, possibly through the expulsion of trichocysts, and quickly transition into a non-motile vegetative form. Trichocysts were

also often seen in bundles near the nucleus. Entire clusters of trichocysts are simultaneously extruded in *Paramecium* when chemically induced or when encountering a predator (Plattner *et al*., 1984; Zieseniss and Plattner, 1985; Knoll *et al*., 1991; Harumoto, 1994). Thus, trichocysts of *Hematodinium* may similarly be expelled synchronously and attach to the host hepatopancreas or other organs. In our *in vitro* life cycle study, *Hematodinium* had developed into dinospores, but since the dinospores did not gain entry into a suitable host within a specific time frame, the parasite likely began to digest trichocysts to conserve energy. Furthermore, if trichocysts serve as an attachment mechanism, it would explain the lack of trichocyst expulsion we observed in culture. If trichocysts are only expelled in order to attach to an organ of the host, then they lacked the necessary stimulus in our *in vitro* conditions. The role of trichocysts at the host-parasite interface has not been investigated in *Hematodinium*. However, it may play a key role in the transmission and ultimate development of the parasite.

Another likely role that trichocysts may play is as a defensive function, once the parasite is outside the host. As mentioned, trichocysts have known roles in predatory defense in ciliates (Knoll *et al*., 1991; Harumoto, 1991). Dinospores are known to survive for relatively long periods outside their host species (Meyers et al., 1987). It seems reasonable to that *Hematodinium* may employ defensive strategies to aid in its survival from predatory protists. As sporont development marks the commitment of cells to dinospore formation, it is possible that they then begin preparation of defensive structures to ameliorate their chance of survival between hosts.

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Chapter 4: CRYOPRESERVATION OF *HEMATODINIUM*

4.1 Introduction

The first successful cryopreservation of human sperm was reported in 1949 (Polge *et al*., 1949), and the ability to freeze cells and tissues has since become a valuable technique for modern biologists and medical personnel. When cells are stored at extremely low temperatures (usually -196°C) metabolic activity essentially halts; thus, the longevity of cells increase as apoptotic activity also stops (Day and Stacey, 2007). This has led to advancements in medicine and biology that would have been impossible: gametes can be preserved and used at a later date, which leads to practical agriculture and human applications (Sanger *et al*., 1992; Bailey *et al*., 2000; Donnelly *et al*., 2001). Cell banks of viruses, prokaryotes, and eukaryotes exist, allowing researchers and manufacturers greater reproducibility when developing products and conducting research. By cryopreserving specimens, researchers are able to mitigate problems associated with conventional methods for the propagation and preservation of microorganisms such as: labour needs, contamination of cultures over time, the loss of original strains, and changes in the original physiological or metabolic characteristics (Mutetwa and James, 1984; Gill and Redwin, 1995; Miyake *et al*., 2004; Rhodes *et al*., 2006; Tanniou *et al*., 2012).

Hematodinium is a parasitic dinoflagellate of numerous crustacean species worldwide. The parasite was first reported in 1931 and has now been reported to infect numerous economically significant species worldwide (Chatton and Poisson, 1931; Newman and Johnson, 1975; Maclean and Ruddell, 1978; Meyers *et al*., 1987; Latrouite *et al*., 1988; Wilhelm and Boulo, 1988; Hudson and Lester, 1994; Hudson and Shields, 1994; Messick, 1994; Taylor and Khan, 1995; Wilhelm and Mialhe, 1996, Ryazanova *et*

al., 2010). Advances in understanding the basic biology of *Hematodinium* are hindered by the difficulty in obtaining a reliable and consistent source of parasites. Higher prevalence has been noted in juveniles (Stentiford and Shields, 2005; Mullowney *et al*., 2011); however, juveniles are not caught using conventional pot trapping techniques and are difficult to obtain (Hoenig and Dawe, 1991; Tremblay, 1997; Mullowney *et al*., 2011). In numerous crab species, those that are of commercial size are less frequently infected (Messick and Shields, 2000; Stentiford and Shields, 2005; Mullowney *et al*., 2011). Furthermore, die-offs caused by *Hematodinium* infections go largely unnoticed because crabs remain on the sea floor when they die. There is also gaps surrounding knowledge of the parasite's life cycle and parameters affecting parasite transmission and subsequent host-parasite interactions. Collectively, these factors reduce the likelihood of obtaining consistent and reliable supplies of *Hematodinium*, and highlight the importance of establishing *in vitro* cultures and the subsequent cryopreservation of cultures in furthering our knowledge of *Hematodinium*. Once isolated, *Hematodinium in vitro* cultures may succumb to contamination or may become senescent and die (Chapter 2; Appleton and Vickerman, 1998; Li *et al*., 2011). Appleton and Vickerman (1998) have reported the sustainability of trophont stages for upwards of five years *in vitro*, but other life stages (such as sporonts and dinospores) appear to be transient lasting only weeks to months in culture (Chapter 2; Appleton and Vickerman, 1998; Li *et al*., 2011). Cryopreservation provides necessary backups for *in vitro* culture required to evaluate transient life stages that would otherwise be missed using primary culture isolations from infected hosts. It is for this reason that the development of cryopreservation techniques for *Hematodinium* was pursued.

Several dinoflagellates have been successfully cryopreserved (Simione and Daggett, 1977; Canavate and Lubian 1997; Rhodes *et al*., 2006; Santiago-Vazquez *et al*., 2007). Common cryoprotectants used to cryopreserve marine microalgae and protozoa are dimethyl sulfoxide (DMSO) and glycerol (Smith *et al*., 1951; Fulton and Smith, 1953; Levine and Marquardt, 1955; Diamond *et al*., 1961; Walker and Ashwood-Smith, 1961; Collins and Jeffery, 1963; Lumsden *et al*., 1966; Callow and Farrant, 1973; Miyata, 1973, 1976; Dumas, 1974; Canavate and Lubian, 1994, 1995; Hubálak, 2003). Since the supply of viable *Hematodinium* was limited for this study, the investigation focused on the suitability of two cryoprotectants, glycerol and DMSO. DMSO was used at a concentration of 10% as this is the median concentration used for cryopreserving many microorganisms (Hubálek, 2003), and glycerol at a concentration of 3% as others have cryopreserved protozoa successfully with low concentrations of glycerol (Miyata, 1973).

4.2 Materials and Methods

4.2.1 Collection of Atlantic snow crabs

Atlantic snow crabs (*C. opilio*) were collected during annual surveys in White Bay, Newfoundland during September 2011. Crabs obtained in 2011 were by crab pot on the grounds of stratum 614 (301-400 m in depth). Water temperatures for stratum 614 normally ranged between 0-1°C in early fall (Mullowney *et al*., 2011). Crabs were kept in coolers layered between seawater soaked burlap, and placed above saltwater ice until reaching shore. On shore, coolers were transported to St. John's, Newfoundland, where they were sent via air cargo to the Atlantic Veterinary College (AVC), University of Prince Edward Island. The time between snow crab harvest and their arrival at AVC was usually 24-48 h. Eight crabs showed morphological changes consistent with Bitter Crab

Disease (BCD). These changes included a discoloured or opaque cuticle and milky white hemolymph.

4.2.2 Necropsy of Chionoecetes opilio and hemolymph collection

Upon arrival, crabs were stored in an aerated holding tank at 0°C with 33-34 ppt salinity for 1-4 h to recover from the stress of shipment. Ice packs were placed in the holding tank in an attempt to keep water temperature at 0°C. Crab viability was assessed by observing mandible or limb movement and response to 70% ethanol sprayed on the mouth and gills. Only live crabs were used in the isolation of dinoflagellates. After carapace width, sex, hemolymph refractive index, and any gross lesions were recorded, crabs were exsanguinated. Hemolymph was collected aseptically from the base of the first periopod using a 3 ml or 5 ml syringe with 22 gauge needle. Hemolymph (200 µl) was placed into each well of a 96 well plate containing 800 µl 80% ethanol for future DNA extraction and genotype confirmation by polymerase chain reaction (as part of separate study). The remaining hemolymph was stored on ice until the completion of all necropsies. After exsanguination, the carapace of the crab was removed, and the heart, hepatopancreas, eyestalk, gills, gonad, gut, and leg muscle were surgically removed and placed in Davidson's seawater fixative for future histopathological examination (as part of Dr. Melanie Buote's Ph.D. research). All necropsies were completed within 3-4 h, depending on the number of crabs obtained in each shipment.

4.2.3 Cryopreservation of Hematodinium cells

After necropsy, the remaining hemolymph containing suspect *Hematodinium* from BCD evident crabs was cultured *in vitro*. Sufficient amounts of hemolymph were only collected from six of eight BCD evident crabs. These were found to be in either the

sporont or trophont life stage (See Chapter 2). Syringes were gently agitated to ensure adequate mixing, and were transferred to microcentrifuge tubes within a Class II biological safety cabinet (Sterigard® III Advance, The Baker Company). Infected hemolymph (40 µl) was added to 1.94 ml of *Nephrops* medium (See Appendix A) in 24 multiwell plates (Falcon) and incubated in a 4.1 ft^3 incubator (Binder-APT line™ KB (E3.1)) at 0 ± 0.2^oC for 24 h. After incubation, samples were processed for cryopreservation. The protocols for cryopreserving cells were adapted from in house protocols.

Briefly, 1 ml of culture was placed in a cryovial. Samples to be cryopreserved using DMSO were centrifuged at 500 X g, then resuspended in a 10% DMSO solution. Cells were frozen at -20°C for 1 h, at -80°C for 2 h, and liquid nitrogen (See Appendix E for more details).

Samples to be cryopreserved using glycerol were added directly to a 3% glycerol solution, placed at -80°C for 24 h, then in liquid nitrogen. (See Appendix F for more details.)

4.2.4 Reviving cryopreserved cultures

Samples were removed from liquid nitrogen after ~3 months. Cryovials were immediately placed on ice water to thaw. After thawing, samples were washed twice by centrifuging at 900 X g for 5 min at 4°C and gently resuspending cells in 1 ml of fresh *Nephrops* medium. After washing, cells were incubated at 0 ± 0.2^oC (Binder-APT.line™ KB (E3.1)). Supernatant collected during washing was kept and pooled, then centrifuged at 900 X g for 10 min at 4°C to recover any cells that may have been lost during previous

washings. The pellet was also resuspended in 1 ml of fresh *Nephrops* medium and incubated at 0 ± 0.2°C.

4.2.5 Hematodinium viability after reviving from cryopreservation

Samples were assessed every 2-3 days for viability by staining with 0.2% Neutral Red (Sigma-Aldrich) or 0.05% Acridine Orange (Sigma-Aldrich). Neutral Red and Acridine orange have previously been used as a diagnostic tool for the study of *Hematodinium*, has widely been used to assess cell viability, and also demonstrates good correlation with other vital stains (Bank, 1988; Geysen *et al*., 1991; Modha *et al*., 1993; Mascotti *et al*., 2000; Soudant *et al*., 2005; Stentiford and Shields, 2005)

One drop of sample (~15 µl) was placed on a glass microscope slide within a class II biological safety cabinet (Sterigard® III Advance, The Baker Company), mixed with an equal volume doubly concentrated vital stain, and viewed immediately on an Axioplan 2 imaging microscope (Zeiss). Samples stained with Neutral Red were viewed with transmitted light, and those stained with Acridine Orange were viewed with ultraviolet light passed through a FITC filter (Zeiss). All images were captured using an Axiocam ACC camera.

4.3 Results

4.3.1 Assessment of viability using vital staining

After reviving cultures following cryopreservation for 3 months, many *Hematodinium* were lysed. There was little difference with regard to survivability and cell morphology of intact cells between cultures cryopreserved in DMSO and glycerol. Viable cells were observed only in one culture (#64). Numerous vacuolated structures resembling micelles or liposomes were noted in all cultures; these did not take up Neutral Red or Acridine

Orange stains (Figure 4.1). Culture #64 had the highest cell density before cryopreservation (Table 4.1). Although parasites were uninucleated and multinucleated prior to freezing, surviving parasites were exclusively uninucleated (Figure 4.2; Figure 4.3). Very few cells survived. Only rarely were viable cells observed using Acridine Orange or Neutral Red stains; thus an accurate survival rate could not be determined. *Hematodinium* from culture #64 were in the trophont stage prior to cryopreserving. Although no ultrastructural data was obtained on cells after thawing, it is presumed that they remained in the trophont life stage. Surprisingly, cells did not seem to replicate; thus, cultures were terminated after 24 days *in vitro*.

4.4 Discussion

Glycerol and DMSO were explored as potential cryoprotectants for *Hematodinium*. Previous attempts to cryopreserve *Hematodinium* isolated from a warm-temperature host, *Callinectes sapidus*, have been unsuccessful (Jeff Shields, personal communication). This is the first reported successful cryopreservation of *Hematodinium* from any host species.

Although the recovery rate of *Hematodinium* was very low, viable cells (as determined with Neutral Red and Acridine Orange staining) were observed. Viable cells were only seen in culture #64, which had the highest cell density prior to cryopreservation; however, these cultures did not seem to replicate. It is possible that the cells could not recover from the initial stress of freezing/thawing and became moribund.

As only few viable cells were observed, higher densities of *Hematodinium* are likely more tolerant to freezing. Another possibility is that because the recovery rate was

Table 4.1: Cell densities associated with six cultures of *Hematodinium* prior to cryopreservation. Of all the samples cryopreserved, viable cells were only apparent in sample #64 after freezing.

Sample	Cell Density (cells/ml)
#61	3.5×10^5
#62	1.5×10^6
#63	5.0×10^4
#64	2.4×10^6
#65	1.45×10^6
#98	2.05×10^6

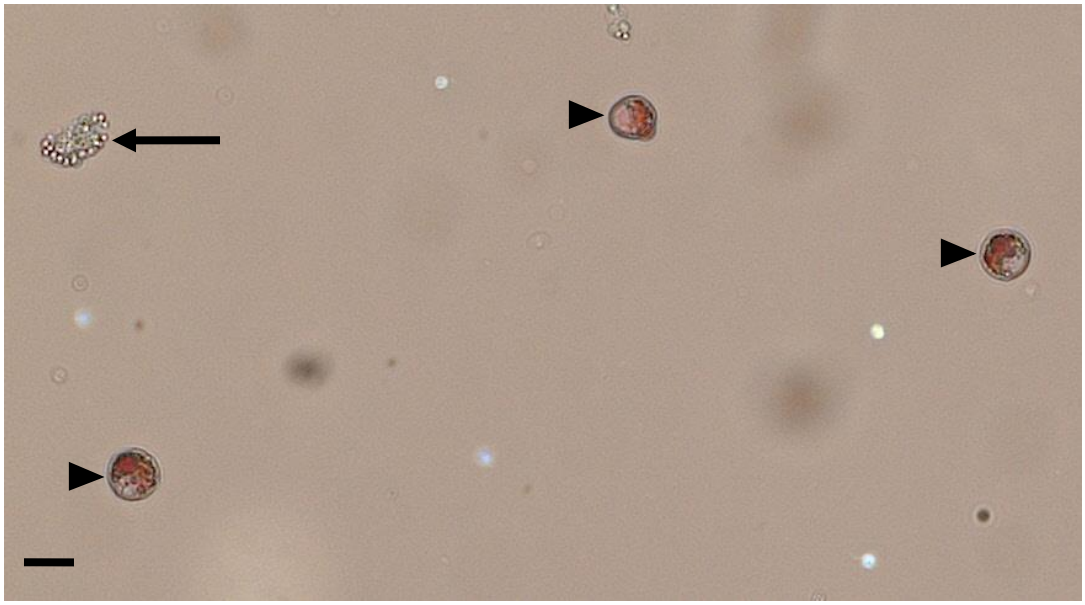

Figure 4.1: Viable and non-viable *Hematodinium* recovered from sample #64 after thawing. Viable cells (arrowhead) were spherical in morphology and took up Neutral Red vital stain. Vacuolated structures (arrow) had no discernable cellular membrane and appeared to be aggregations of liposomes or micelles. Scale bar: 20 µm.

Figure 4.2: Viable *Hematodinium* recovered from sample #64 four days after thawing and stained with Acridine Orange. Nuclear material fluoresced green and acidic organelles, such as lysosomes, fluoresced orange when excited with UV light. Cells were uninucleated and had several acidic organelles. Scale bar: 20 μm.

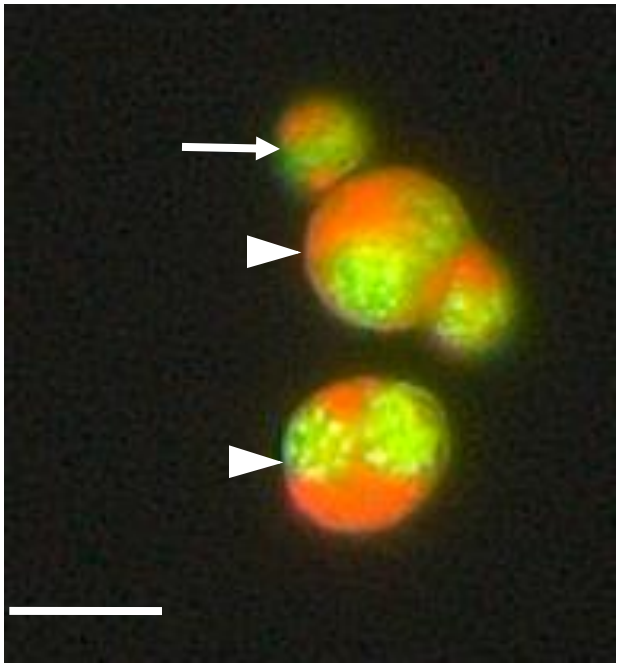

Figure 4.3: Viable *Hematodinium* from a non-cryopreserved sample stained with Acridine Orange four days *in vitro*. Nuclear material fluoresced green and acidic organelles, such as lysosomes, fluoresced orange when excited with UV light. In contrast to cryopreserved specimen, *Hematodinium* were uninucleated (arrow) and multinucleated (arrowheads). Scale bar: 20 µm.

so low, we were unable to detect viable cells in cultures with lower densities with our current methods. Debris in the culture is believed to be the result of lysed cells, which lysed either at the time of freezing, during thawing, or during centrifugations. When cells freeze without adequate cryoprotection, ice crystals form in the cytoplasm which leads to cell death (Trump *et al*., 1964; Whittaker, 1974, 1984). Glycerol and DMSO are usually adequate for cryoprotecting cells as they easily permeate cell membranes, thereby increasing the osmolarity of the cell, making the cell cytoplasm harder to freeze; however, the rate at which penetration occurs largely depends on temperature and cell type (Hubálek, 2003). Furthermore, although cells may survive freezing, they are still fragile at the time of thawing and shortly thereafter, as they have undergone severe stress (Fuller and Bernard, 1984; Fuller and Paynter, 2004). Generally, dinoflagellates tend to be less viable after freezing than other unicellular algae (Day, 2007); the reason for this phenomenon is not well understood. The life and growth stage of the organism likely plays a significant role in the success of cryopreservation (Ishikawa et al., 2006; Baydoun, 2010). The life stage of the organism was documented prior to freezing (for life stages from crab hemolymph see Chapter 2); however, the growth phase (i.e. log, lag, and stationary phases) could not be determined, as cultures were cryopreserved after only 24 h in culture. Additionally, *Hematodinium*, unlike most dinoflagellates, do not have thecae. Thecae are generally, very robust cell wall plates and serve as added protection to the organism. Because *Hematodinium* lack these, their membranes are more exposed and susceptible to mechanical disruption than thecated dinoflagellates. Thus, freezing, thawing, or centrifugation after thawing may have destroyed the integrity of cell membranes making them fragile. Perhaps a gentler mode of cryopreservation is needed

than typical methods for cryopreserving dinoflagellates. Increased recovery of cells is likely if centrifugation did not proceed immediately after thawing as mechanical disruption may have sheared cells. Centrifugation of *Hematodinium* and subsequent resuspension in fresh media was performed due to the toxic nature of most cryoprotectants, especially DMSO (Brayton, 1986). Placing *Hematodinium* in fresh culture medium for at least 24 hours before centrifugation may give the organism time to acclimate to the warmer temperature, would dilute the cryoprotectant, and allow time for repairing any cellular damage that may have occurred during freezing/thawing.

There appeared to be many vacuolated structures appeared cell-like, but were found to be non-viable. What were thought to be vacuoles may have been liposomes or micelles that naturally developed once the cell membrane lysed. Although the cell membrane lysed, the amphiesmal membranes appeared to stay intact in most cases, thus retaining any liposomes or micelles that may have formed. Although many cells did not survive freezing and reviving, some *Hematodinium* trophonts did survive in culture for 24 days without replication, giving evidence of the parasite's resiliency and tolerance to extreme temperatures.

Future cryopreservation experiments may yield better results if different cryoprotectants are used or the existing cryoprotectants are used at different concentrations. One would have to assess the tolerance of *Hematodinium* to increased concentrations of DMSO or glycerol as they can be toxic (Brayton, 1986; Katkov *et al*., 1998; Hubálek, 2003); however it is likely that the parasite may survive in higher concentrations of each cryoprotectant as DMSO has been used up to 32% (v/v) and the median use of glycerol is 10% 0 (v/v; Miyata, 1975; Hubálek, 2003).

Hematodinium did not show any evidence of replication or growth after being revived and eventually lost viability. The life stage prior to cryopreservation likely played a role in survivability. Cultures were either frozen in the trophont or sporont life stage. One culture had viable cells after freezing; it was in the trophont life stage prior to freezing. Cryopreservation of *Hematodinium* in the dinospore life stage was not attempted. Furthermore, growth phase likely plays an important role in survivability post freezing, as in other cell types (Ishikawa *et al*., 2006; Baydoun, 2010). Usually cells in log phase are cryopreserved more effectively. For this reason, cells were placed in culture for 24 h before freezing; however, growth phase and growth parameters of *Hematodinium* were not determined prior to freezing. A longer acclimation period to culture medium may prove beneficial to cryopreservation. Furthermore, based on *in vitro* work (Chapter 2) trophonts did not progress to further stages. Thus there seems to be something lacking in culture conditions to stimulate *Hematodinium* growth and development in that particular stage. Adding crab hemolymph or other nutrients to mimic natural conditions may aid in *Hematodinium* growth and development post-freezing.

Additionally, the cells may be incapable of overcoming the initial stress of low temperature after thawing and centrifugation. A second possibility is that *Hematodinium* will only grow and replicate when it is in close proximity to other viable cells. Filopodia-like structures could play a role in cell to cell communication (discussed in Chapter 2). *Hematodinium* replicate to incredible densities *in vivo* (Stentiford and Shields, 2005), but the number of cells needed to cause infection is unknown. It is possible that close proximity of *Hematodinium* to neighbouring cells are needed for growth and replication

to occur. There was much debris in cultures with very few cells; thus, seeding density was perhaps inadequate to establish cultures *in vitro*.

Notably, only uninucleated cells were viable after freezing, even though multinucleated cells were present in culture prior to freezing. This suggests that the life stage and/or metabolic activity of *Hematodinium* prior to freezing is important in determining the success rate of cryopreservation. Cells that were viable were from sample #64 and in the trophont life stage prior to freezing based on ultrastructural analysis. Although trophonts are uninucleated and multinucleated, they are seen more often as multinucleated cells (Appleton and Vickerman, 1998; Chapter 2), which may account for the low recovery rates. Uninucleated cells are presumed to be less metabolically active; they are not in the process of cell division. Perhaps *Hematodinium* are more tolerant to freezing when less metabolically active. This is contrary to other findings, where cells in log phase are generally suitable for cryopreservation (Day and Stacey, 2007).

In summary, this preliminary study is the first successful attempt at cryopreserving and reviving *Hematodinium* from any host species, and highlight *Hematodinium*'s potential for long-term storage. The cells that survived were uninucleated, and uniformly spherical; however, they lost viability over time. Future experiments may be improved by several means: investigating different cryoprotectants, investigating different concentrations of cryoprotectants, investigating the role of life stages and growth phase and modifying the thawing procedure.

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Chapter 5: GENERAL CONCLUSIONS

5.1 Developmental stages

Throughout this investigation, the principal hypothesis was that similar life stages as those described by Appleton and Vickerman (1998) and Li *et al*. (2011) would be observed in *Hematodinium* isolated from Atlantic snow crab, *Chionoecetes opilio*. The results indicate that similar stages are apparent, namely amoeboid trophonts, coenocytes, sporonts, and dinospores. Furthermore, aberrant forms such as the schizont were observed. Unlike Appleton and Vickerman (1998) and Li *et al*. (2011) gorgonlocks colonies or viable clump colonies were not observed.

To observe these stages, sustainable cultures were needed. This objective was successfully accomplished. Several *in vitro* media were assessed and *Nephrops* medium yielded superior results. There were several instances of bacterial and/or fungal contamination, however once discovered, mitigation steps were performed immediately.

Characterization of stages was performed on cultures using scanning and transmission electron microscopy and light microscopy. Thus, ultrastructure analysis was performed on *in vitro* cultures in addition to light microscopical analysis. Using electron microscopy, stages with dissimilar morphology (i.e. amoeboid trophonts) showed similar cell ultrastructure. Thus no distinction between filamentous and amoeboid trophonts was observed. Conversely, cells that had similar morphology (i.e. some amoeboid trophonts and sporonts) had dissimilar ultrastructure. Sporonts have trichocysts whereas trophont stages do not. Ultrastructurally, all stages contained nuclei with permanently condensed chromosomes, lipid droplets, lipofuscin granules, amphiesmal vesicle membranes, and mitochondria with tubular cristae. Trophonts contained solely these structures; sporonts possessed these structures as well as trichocysts. The

appearance of trichocysts was characteristic of sporonts. Dinospores possessed trichocysts as well as two flagella. The appearance of flagella was characteristic of dinospores.

Cultures in which sporonts were observed, further progression to dinospore stages was noted. Two types of coenocytes were also observed in association with sporonts: sheet-like coenocytes and arachnoid coenocytes. In cultures with trophont stages, there was no progression to dinospores. Furthermore, only one type of coenocyte (sheet-like coenocytes) was noted in association with trophonts. In some cultures, *Hematodinium* remained as amoeboid trophonts for the duration of monitoring, which was four months. In cultures that did progress to dinospores, progression took approximately one month, which is comparable to previous *in vitro* studies of *Hematodinium* (Appleton and Vickerman, 1998; Li *et al*., 2011) days. Notably, only macrodinospores were observed, but this is likely due to the low number of cultures obtained. After ~ 2 weeks dinospores lost their flagella and became spherical in morphology. Furthermore, their nuclei were notably different than other stages. Thus they were described as 'post-dinospores'. Transition from dinospores to other stages may be partly time-dependent. Thus, it is possible that dinospores began transition, but lacked the chemical and/or environmental cues to successfully complete transition to the next life stages. Additionally, schizonts similar to those described by Li *et al.* (2011) were seen. These were considered aberrant forms that appeared when cultures were old or were contaminated.

5.2 Cryopreservation

The secondary hypothesis during this investigation was that *Hematodinium*, being from a cold water host, would be able to survive cryo-freezing. Two cryoprotectants were used: 3% glycerol and 10% DMSO, both of which yielded positive results. Some *Hematodinium* from the densest culture survived cryoprotection for three months. Although the recovery rates were very

low, this is the first successful attempt at cryopreserving *Hematodinium* from any species. Cryopreserving *Hematodinium* would be valuable for future research as one would have backup cells for future culture initiation should one wish to perform additional testing or should an existing culture become contaminated and/or die. Additionally, one would have consistent supply of *Hematodinium* needed for infection studies or any other studies one may wish to pursue.

5.3 Notable ultrastructural features

During the current investigation, certain features of *Hematodinium*'s ultrastructure were intriguing. Most notable was the trichocyst. The trichocyst was a rectangular organelle, bounded by a single membrane and was square in cross-section. It contained two main parts: the body and the apical end. The body seemed rigid and very electron dense. It consisted of a crystalline core, presumably of proteinaceous origin. The apical end (the region between where the crystalline core ends and the end of the trichocyst, near the cell membrane) was more complex. It contained numerous tubules that attached to cuboid appendages situated on the apex of the crystalline core. The other ends of tubules attached part way up the apical end. Additionally, electron dense hoops lined the periphery of the apical end. Trichocysts developed non-synchronously within cells. They began as primordial vesicles associated with the trans-face of the Golgi, where they dissociated and the crystalline core began to polymerize. As the crystalline core condensed, an electron lucent area between the core and the trichocyst membrane was generated. Mature trichocysts were characterized when the trichocyst membrane was tightly apposed to the core. Furthermore, trichocysts may be degraded in autophagocytic vesicles, where striations appeared on the crystalline core.

Trichocysts were only observed intracellularly and never as extruded into the extracellular space. *Hematodinium* was likely not exposed to the necessary environmental and/or chemical stimuli to promote extrusion. Further research investigating the mechanism of trichocyst expulsion is required to determine the role that trichocysts play in *Hematodinium*'s life cycle. Furthermore, investigations into the nature of trichocyst components would be useful in determining the overall structural dynamics of trichocysts.

Another interesting ultrastructural feature that was apparent in all *Hematodinium* cells were large, irregular shaped, vesicles with heterogeneous granules, which autofluoresced. These vesicles have similar characteristics to lipofuscin found in vertebrates and invertebrates (Gordon *et al*., 1965; Boellaard and Schlote, 1986; Sarna *et al*., 2003; Vogt, 2012). This is the first time lipofuscin has been reported in *Hematodinium*. Similar vesicles have been noted in other *in vitro* studies (Appleton and Vickerman, 1998), but have not been characterized. Lipofuscin is probably derived from the diet of *Hematodinium* since low levels of hemocyanin have been noted in association with *Hematodinium* infection, *Hematodinium* may actively ingest hemocyanin, and the Cu^{2+} may trigger lipofuscin formation in lysosomes. In some trophonts lipofuscin granules represented the majority of the cellular volume and thus it seems apparent that these structures are an important component of *Hematodinium*.

Additionally, lipofuscin appeared to be degraded or removed from the cell over time. As cells were sustained in culture, lipofuscin granules began to dissipate, leaving a large electron lucent area in the middle of the vesicle. How this dissipation occurred is unknown, but could either be through active digestion or piecemeal degradation. Further studies investigating the mechanism of lipofuscin degradation in *Hematodinium* would be worthwhile as lipofuscin comprised a large portion of the cell. Furthermore, lipofuscin is purportedly undegradable

(Terman and Brunk, 2004), thus knowledge of how *Hematodinium* purges this substance could be valuable to a wide range of researchers. Additionally, little knowledge of the components of lipofuscin granules in *Hematodinium* is known. As lipofuscin was abundant in *Hematodinium* cells, characterizing the chemical nature of lipofuscin contents would be valuable to better understand the biochemistry and physiology of *Hematodinium*.

Filopodia-like structures derived from *Hematodinium* were also observed. These were often several times the length of individual cells, and were seen attached to neighbouring cells. Furthermore, when cells were vital stained, filopodia-like structures seemed to flutter in the medium, but quickly retracted in presence of the direct fluorescent light. The role these filopodia-like structures play in *Hematodinium* is not understood; roles in cell to cell communication, cell motility, or transmission remain possibilities.

5.4 References

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Appendix A

Preparation of *Nephrops* Medium

Ingredients per 1 L of Medium:

NaCl (Omni-Pur-7710)	27.99 g
KCl (Sigma-P5405)	0.950 g
$CaCl_2$ (Omni-Pur-3000)	2.014 g
$MgSO_4$ (Alfa Aesar L13739)	2.465 g
Na_2SO_4 (Sigma-238597)	0.554 g
HEPES Buffer (Omni-Pur-5320)	1.920 g
Distilled Water	883 ml

Adjust pH to 7.8 ± 0.1

Autoclave at 121 °C for 45 min

After the above has been autoclaved,
Aseptically add:

Heat Inactivated Fetal Bovine Serum (PAA-Cat No: A15-704)	100 ml
Gentamicin Stock solution (12000 µg/ml) (Sigma-G1264)	16.67 ml

Keep medium at 4 °C

Appendix B

Preparation of Modified Ciliate Medium

Ingredients per 5 L of stock medium:

Proteose peptone **(**Difco 0120-01)	50 g
Bacto-Tryptone (Difco 0123-01)	50 g
Yeast RNA (Sigma R-6625)	5 g
Distilled water	500 ml
Filtered artificial seawater (Instant Ocean)	4000 ml

Ingredients to be added to 450 ml of above before use:

Heat inactivated Fetal Bovine Serum **(**Gibco 12483-020)	50 ml
RPMI 1640 vitamins (Sigma R-7256)	1 ml
Penicillin/Streptomycin solution	5 ml

(Gibco 15140-148, 10000 units/ml penicillin, 10000 µg/ml streptomycin)

Procedure to prepare 5 liters of medium (stock stored at 4 °C):

1. Dissolve the proteose peptone, bacto-tryptone and yeast RNA in artificial seawater at room temperature (20-22 °C).

2. Place medium on heated stir plate and heat to 80 °C until all ingredients are dissolved.

3. Place medium in 1 L medium bottles (do not fill above 700 ml mark). Loosely cap bottles and autoclave for 25 min at 121 °C.

4. Aseptically aliquot 450 ml of ciliate culture medium to 500 ml medium bottles and store at 4 °C.

5. Before use, add to 450 ml of medium:

   50 ml of heat-inactivated fetal bovine serum
   1 ml of RPMI vitamin solution
   5 ml of penicillin/streptomycin.

Appendix C

Preparation of Dulbecco's modified Eagles medium: Hams F12 medium

For 1.2 L solution:

1) Add 3.76 g of Dulbecco's modified Eagles medium (Sigma-Cat No: D5523) to a total of 376 ml Artificial Sea Water (33ppt) making a 10g/L solution
2) Add 376 ml to 752 ml of Hams F12 solution (Lonza-Cat No: 12-645F) in a 2 L beaker.

To the above solution add:

HEPES (Omni-Pur- Cat No: 5320)	28.06 g
$NaHCO_3$ (Fisher- Cat No: S233-1)	0.706 g
Glucose (Sigma- Cat No: G5767)	3.423 g
Penicillin/Streptomycin (10000 U/ml) (Gibco-Cat. No: 15140)	12 ml
Heat Inactivated Fetal Bovine Serum (PAA-Cat No: A15-704)	60 ml

Adjust pH to 7.4
Filter sterilize solution using an 0.20 µm filter (Sarstedt- Cat No: 83.1826.001)

Appendix D

Preparation of Epon Resin

In a 250 ml beaker mix:

20 ml Araldite (Canemco)
25 ml Jembed (Canemco)
60 ml Dodecenyl succinic anhydride (Canemco)

Stir by hand with wooden applicator for ~2 min.
Add magnetic stir bar to solution, seal with parafilm to prevent moisture contamination, and stir for 30 min.
Remove parafilm, and add 2.4 ml 2,4,6,-Tri-Dimethylaminoethyl)phenol.
Reseal with parafilm and stir with magnetic stir bar for 30 min.
Remove parafilm, and place beaker containing Epon solution in vacuum desiccator for 1 h to degas and desiccate.
Transfer solution into 10 ml syringes.
Store syringes in freezer (-4 ^{o}C)

Appendix E

Cryopreservation of Hematodinium using 10% DMSO

1) Mix culture several times with pipette to ensure cells detached from culture wells.

2) Draw a 1 ml aliquot and transfer aseptically in cryovials (VWR). Perform this within a Class II biological safety cabinet (Sterigard® III Advance, The Baker Company).

3) Centrifuge cell suspension at 500 X g for 5 min at 4 °C.

4) While centrifuging, prepare DMSO solution as follows:

Add 7.5 ml of artificial sea water (ASW) (Instant Ocean; 33 ppt) to 2.5 ml distilled water (dH_2O) to obtain a 75% ASW solution.

Sterilize 75% ASW solution by passing through a 0.2 µm filter (Sarstedt) into a sterile microcentrifuge tube. This should be performed in a biological safety cabinet.

Aseptically add 200 µl DMSO (Sigma-Aldrich-D2650) to 1 ml of sterile 75% ASW solution to make a 20% DMSO solution.

5) After centrifugation, remove most of the supernatant, leaving the cell pellet and approximately 200 µl of solution.

6) Resuspend cells.

7) Add 200 µl of 20% DMSO solution to cell suspension.

8) Invert cryovial approximately five times or until solution has been thoroughly mixed.

9) Incubate cryovial at -20 °C for 1 h.

10) Transfer cryovial to -80 °C incubator and incubate for 2 h.

11) Quickly transfer cryovial to liquid nitrogen for storage.

Appendix F

Cryopreservation of Hematodinium using 3% Glycerol

1) Mix culture several times with pipette to ensure cells detach from culture wells.

2) Draw a 1 ml aliquot of suspended culture medium and transfer aseptically into a cryovial. Perform this within a Class II biological safety cabinet (Sterigard® III Advance, The Baker Company).

3) Add 30 µl of 100% glycerol (Fisher Scientific-G33) to the cell suspension.

4) Mix the suspension by pipette until adequately mixed and until no remaining glycerol remains in the pipette tip.

5) Incubate cryovial at -80^{o}C for 24 h.

6) Quickly transfer cryovials into liquid nitrogen storage for storage.